



Microbial degradation of keratin-rich porcine by-products for protein hydrolysate production: a process engineering perspective

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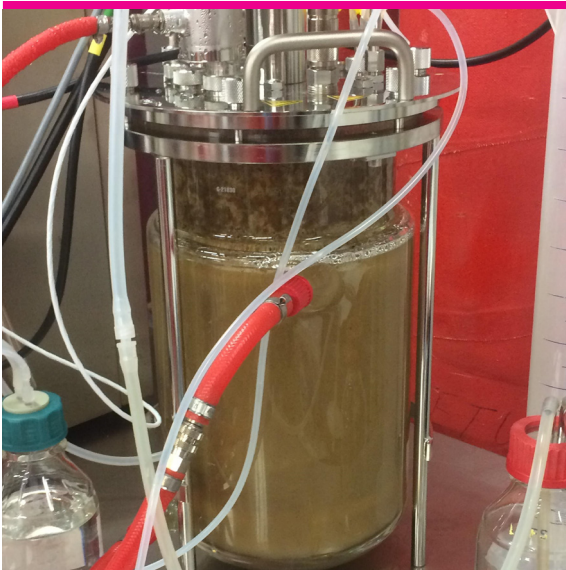
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Microbial degradation of keratin-rich porcine by-products for protein hydrolysate production: a process engineering perspective



Francesco Cristino Falco

PhD Thesis

May 2018



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Ph.D. Thesis

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May 2018

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Preface

This PhD thesis concludes my work at the Department of Chemical and Biochemical Engineering, Technical University of Denmark, from October 1st 2014 to April 30th 2018. The work has been conducted under the Keratin2Protein project, a research financed by the Danish Strategic Research Council | The Programme Commission on Health, Food and Welfare (Grant No. 1308-00015B).

First of all, I would very much like to thank my main supervisor Krist V. Gernaey and co-supervisor Anna Eliasson Lantz for giving me the opportunity to pursue my PhD studies here at DTU, for their excellent guidance, constant inspiration, valuable and constructive discussions, ongoing support, and patience throughout the whole period of my thesis.

I would like also to thank all my wonderful PROSYS colleagues (ex. CAPEC-PROCESS) for sharing this DTU adventure with me and in particular Carolina, Dasha, Eduardo Stefano, Soheil, Tannaz. It has been an honor to be part of such a fantastic group of colleague and friends. A special thank also to my office mates Katrin and Chitta; despite my tendency to bring chaos in each environment I colonize and my passion for covering every inch of available surface with my stuffs they bear my presence for all this time without any complain and always with a smile.

I especially would like to thank Roall Espersen and Birte Svensson from DTU Bioengineering - Enzyme and Protein Chemistry, Yuhong Huang and Lene Lange from DTU Chemical Engineering - BIOENG group, Troels Samuelsen from DAKA and Kim Schøn Ekmann Biomar for their precious collaboration and technical support.

A special thank goes to Gitte Læssøe and Eva Mikkelsen for taking care of all PhD students, Postdocs and Professors in our centre. Thanks a lot for the constant help and personal assistance during these three years here at PROSYS.

I am also grateful to my parents for all their support and love and to my two “little” brothers that are now like me spread around the world. And lastly I must thank my beloved spouse Çiğdem for the infinite patience that she had while I was constantly running experiments during the weekend (sometimes staying also the night in DTU) and for the immeasurable happiness that she brings into every day of my life spent together and that keeps me going forward even when, sometimes, things seem to become more a little bit more difficult.

Brain: Pinky, we must prepare for tomorrow night.

Pinky: Gee, Brain, why? What are we going to do tomorrow night?

Brain: The same thing we do every night, Pinky - try to take over the world!

From the Pinky and the Brain series

Abstract

Keratinous animal by-products such as chicken feathers, horns, wool, hairs, bristles etc. are naturally occurring polymeric materials that are normally synthesized within the epithelial cells of higher vertebrates and humans as well, and constitute after cellulose and chitin the third most abundant renewable biomass present on the surface of our planet. Keratin waste materials occur abundantly in slaughterhouses and meat & poultry processing plants, and according to the EU (Regulation (EC) No. 1096/2009) they are classified under the category of the low-risk animal by-products. As a consequence, this type of solid residue is not suitable for human consumption and needs to be treated before its disposal into the environment. In this PhD thesis we have focused our attention primarily towards a particularly underestimated and therefore underexploited keratin-rich by-product, namely pig bristles (also known as hog hairs). As a matter of fact, the protein content of porcine bristle can be even larger than 80% of its dry weight. Moreover, considering that in 2014 about 250 million pigs were reared and slaughtered in the EU alone (EUROSTAT, 2016), it means that potentially approximately 60 k-tons of dry proteins could be recovered from this solid waste and then be employed as a renewable and more sustainable source of animal feed proteins. Nevertheless, the conventional methods employed for converting pig bristles into a more digestible dietary protein are normally based on the hydrothermal cooking with or without the addition of acid or alkali. The resulting product, namely hog hair meal, is usually lacking sufficient essential amino acids, (methionine, histidine, tryptophan and lysine among others) and still characterized by a relatively low digestibility. Therefore, it would be very beneficial if one could develop a milder technology, which could be utilized to effectively recover the soluble proteins, peptides and amino acids entrapped within this hair-type keratin waste and which, at the same time, could preserve an unaltered nutritional value of the extracted macronutrients. A solution to this problem can be found in the biotechnological route where instead microorganisms and microbial keratinolytic enzymes are employed to bring about the decomposition of keratin-rich substrates. The microbial hydrolysis of the keratinous biomass is a conversion process in which a biocatalytic cocktail of keratin-specific proteolytic enzymes is secreted by bacteria, actinomycetes and keratinophilic fungi that are capable of using keratin as their only source of C, N and energy. The soluble proteins, peptides and free amino acids released during the biological degradation process could constitute a viable alternative protein source to fish meal for aquaculture.

Therefore the main objectives of this PhD thesis are an investigation of the capability of a keratinolytic microorganism, i.e., the filamentous bacterium *Amycolatopsis keratiniphila* D2, to direct the

efficient biodegradation of thermally pretreated pig bristles and the development of a biotechnological process to bring about the cost-effective microbial conversion of porcine bristles into a protein-rich keratin hydrolysate which could then be included in fish feed formulations as an alternative protein source for aquaculture.

In Chapter 2 the effect of culture conditions on bristle protein hydrolysate production during microbial decomposition of thermally pretreated porcine bristles by *A. keratiniphila* D2 was investigated. The maximum concentration of total extracted proteins (i.e. crude soluble proteins + NH_2 -free amino groups) in a 3-L aerobic fermenter was obtained for a degradation process carried out at a cultivation temperature of 35 °C, an initial pH of 6.9, a pig bristle concentration equal to 7% (w/w) and an inoculum grown in GYM (glucose, yeast extract, malt extract) medium for 48 hours. The microbial single-stage hydrolysis process resulted in a final concentration of total extracted proteins of 27 $\text{g}\cdot\text{L}^{-1}$ (45.8 % overall yield of extraction). In Chapter 3, it was demonstrated that a novel two-stage fermentation process, which was additionally run at high solids loadings (15% w/v), could result in a very significant improvement in the amount of total extracted proteins (98 $\text{g}\cdot\text{L}^{-1}$; overall extraction yield of 84.9%) when hydrolyzing thermally pretreated porcine bristles with *A. keratiniphila* D2. Afterwards, in Chapter 4, the use of cell-free crude keratinases extract obtained from *A. keratiniphila* D2 was tested as an alternative to direct the enzymatic degradation at high solids loadings (15% w/w) of thermally pretreated porcine bristles in the absence of microbial cells. Moreover, two different fed-batch keratinous waste hydrolysis strategies were tested. In addition, the nutritional quality of the obtained keratin protein hydrolysates was also evaluated which, indeed, was confirmed to be improved considerably with respect to the original keratinous material. Finally, Chapter 5 provided a more detailed investigation of some of the proteolytic enzymes which were synthesized by *A. keratiniphila* D2 when grown on keratinous by-products. In particular, two different proteases were purified from the culture supernatant and were further characterized: both proteolytic enzymes were shown to belong to the S1 family of proteases and to have higher specificity towards keratin-rich substrates.

In conclusion, this PhD thesis provides for the first time, a systematic insight into the development of a biotechnological process of industrial relevance for the recovery of valuable proteins from an exceptionally recalcitrant hair-type keratinous by-product, that is porcine bristles.

Resumé på Dansk

Keratinholdige animalske biprodukter såsom kyllingfjer, horn, uld, hår, børster osv. er naturligt forekommende polymere materialer, som normalt syntetiseres i epithelcellerne hos højere hvirveldyr og mennesker, derudover udgør de efter cellulose og chitin den tredje mest tilgængelige og bæredygtige biomasse til stede på vores planet. Keratinaffaldsmaterialer forekommer rigeligt i slagterier og kød- og fjerkræforarbejdningsanlæg, og i henhold til EU (forordning (EF) nr. 1096/2009) er de klassificeret under kategorien lavrisikoprodukter af animalske biprodukter. Som følge heraf er denne type faste restprodukter ikke egnet til indtagelse og skal forarbejdes, før dens bortskaffelse i miljøet. I denne ph.d.-afhandling har vi primært fokuseret på et særligt undervurderet og derfor underudnyttet keratinrigt biprodukt, nemlig svinebørster. Faktisk kan proteinindholdet i svinebørster være endnu større end 80% af dets tørvægt. I betragtning af, at der i 2014 kun er opdrættet og slagtet 250 millioner svin i EU alene (EUROSTAT, 2016), betyder det, at potentielt ca. 60 kilotons tørre proteiner kunne udvindes fra dette faste affald og derefter blive anvendt som en vedvarende og mere bæredygtig kilde til dyrefoderproteiner. Ikke desto mindre er de konventionelle metoder, der anvendes til omdannelse af svinebørster til et mere fordøjeligt kostprotein, normalt baseret på den hydrotermiske kogning med eller uden tilsætning af syre eller alkali. Det resulterende produkt, nemlig svinehårsmel, mangler sædvanligvis tilstrækkelige essentielle aminosyrer, (methionin, histidin, tryptophan og lysin blandt andre) og er stadig kendetegnet ved en relativt lav fordøjelighed. Derfor ville det være meget gavnligt, hvis man kunne udvikle en mildere teknologi, som kunne anvendes til effektivt at genoprette de opløselige proteiner, peptider og aminosyrer som er fanget i disse hårkeratinaffald, og som samtidig kunne bevare en uændret næringsværdi af de ekstraherede makronæringsstoffer. En løsning på dette problem kan findes i den bioteknologiske vej, hvor der i stedet anvendes mikroorganismer og mikrobielle keratinolytiske enzymer til at forårsage nedbrydning af keratinrige substrater. Den keratinholdige biomasses mikrobielle hydrolyse er en konverteringsproces, hvori en biokatalytisk cocktail af keratinspecifikke proteolytiske enzymer udskilles af bakterier, actinomyceter og keratinofile svampe, der er i stand til at anvende keratin som deres eneste kilde til C, N og energi. De opløselige proteiner, peptider og frie aminosyrer frigivet under den biologiske nedbrydningsproces kunne udgøre en brugbar alternativ proteinkilde til fiskefodde til akvakultur.

Derfor er hovedformålene med denne ph.d.-afhandling en undersøgelse af evnen af en keratinolytisk mikroorganisme, dvs. den trådformede bakterie *Amycolatopsis keratiniphila* D2, til at lede den effektive bionedbrydning af termisk forbehandlede svinebørster og udviklingen af en bioteknolo-

gisk proces for at skabe effektive mikrobiel omdannelse af svinebørster til et proteinerigt keratinhydrolysat, som derpå kunne indgå i fiskefoderformuleringer som en alternativ proteinkilde til akvakultur.

I kapitel 2 bliver effekten af kulturbetingelser på børsteproteinhydrolysatproduktion under mikrobiel nedbrydning af termisk forbehandlede svinebørster af *A. keratiniphila* D2 undersøgt. Den maksimale koncentration af total ekstraherede proteiner (dvs. råopløselige proteiner + NH_2 -fri aminosyrer) i en 3-L aerob fermenter blev opnået til en nedbrydningsproces udført ved en dyrknings-temperatur på 35 °C, en initial pH på 6,9, en svinebørstekoncentration svarende til 7% (vægt / vægt) og et inokulum dyrket i GYM (glukose, gærekstrakt, maltekstrakt) medium i 48 timer. Den mikrobielle en-fase hydrolyseproces resulterede i en endelig koncentration af totalt ekstraherede proteiner med $27\text{ g}\cdot\text{L}^{-1}$ (45,8% samlet udvindingsudbytte). I kapitel 3 blev det påvist, at en ny to-trins fermenteringsproces, der desuden kørte ved belastninger med høj faststofindhold (15% vægt / volumen), kunne resultere i en meget betydelig forbedring af mængden af samlede ekstraherede proteiner ($98\text{ g}\cdot\text{L}^{-1}$; samlet ekstraktionsudbytte på 84,9%) ved hydrolysering af termisk forbehandlede svinebørster med *A. keratiniphila* D2. Derefter blev anvendelsen af cellefri råkeratinaserekstraktion opnået fra *A. keratiniphila* D2 testet som et alternativ til at styre den enzymatiske nedbrydning ved høje faststofbelastninger (15% vægt / vægt) af termisk forbehandlede svinebørster i fravær af mikrobielle celler. Desuden blev to forskellige fed-batch keratinholdige affaldshydrolysestrategier testet. Derudover blev næringskvaliteten af de opnåede keratinproteinhydrolysater også vurderet, hvilket faktisk blev bekræftet i at være forbedret betydeligt i forhold til det oprindelige keratinholdige materiale. Endelig bringer kapitel 5 en mere detaljeret undersøgelse af nogle af de proteolytiske enzymer, der blev syntetiseret af *A. keratiniphila* D2, når de blev dyrket på keratinbiprodukter. Især blev to forskellige proteaser oprenset fra kultursupernatanten og blev yderligere karakteriseret: begge proteolytiske enzymer blev vist at tilhøre S1-familien af proteaser og have højere specificitet over for keratinrige substrater.

Afslutningsvis giver denne ph.d.-afhandling for første gang en systematisk indsigt i udviklingen af en bioteknologisk proces af industriel relevans til nyttiggørelse af værdifulde proteiner fra et usædvanligt genstridigt keratinbiprodukt af svinebørster.

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List of Publications

Paper I

Francesco Cristino Falco, Krist V. Gernaey. An experimental design for the optimal biodegradation of pretreated pig bristles by the keratinolytic actinomycetes *Amycolatopsis keratiniphila* D2. Manuscript in preparation.

Paper II

Francesco Cristino Falco, Roall Espersen, Birte Svensson, Krist V. Gernaey, Anna Eliasson Lantz. An integrated strategy for the cost-effective production of bristle protein hydrolysate by the keratinolytic filamentous bacterium *Amycolatopsis keratiniphila* D2. Manuscript in preparation.

Paper III

Francesco Cristino Falco, Roall Espersen, Birte Svensson, Anna Eliasson Lantz, Krist V. Gernaey. Bioconversion of pretreated porcine bristles into protein-rich hydrolysates for aquafeed by the keratinolytic actinobacterium *Amycolatopsis keratiniphila* D2. Manuscript in preparation.

Paper IV

Roall Espersen, Francesco Cristino Falco, Per Hägglund, Krist V. Gernaey, Anna Eliasson Lantz, Birte Svensson. Characterization of two novel proteases associated with the ability of *Amycolatopsis keratiniphila* to degrade keratinous slaughterhouse by-product. Manuscript in preparation.

Conference Proceedings

Oral Presentation

Enhanced microbiological degradation of hard keratins by *Amycolatopsis keratiniphila* D2 for protein hydrolysates production. 1st International Conference on Bioresource Technology for Bioenergy, Bioproducts & Environmental Sustainability, Sitges, Spain, 23-26 October 2016.

Abbreviations

ANOVA analysis of variance

BHT butylated hydroxytoluene

BPH bristle protein hydrolysate

BPH_A bristle protein hydrolysate A

BPH_B bristle protein hydrolysate B

BV biological value

CCD central composite design

CSH culture supernatant hydrolysate

DoE design of experiments

EQ ethoxyquin

FM feather meal

GTP guanosine triphosphate

IF intermediate filament

LB Luria-Bertani

NADH nicotinamide adenine dinucleotide

OVAT one-variable-at-a-time

PBM porcine bristle meal

PDCAAS digestibility-corrected amino acid scoring

PER protein efficiency ratio

RSM response surface methodology

SEC size exclusion chromatography

SmF submerged fermentation

SSF solid-state fermentation

StgA feeding strategy A

StgB feeding strategy B

WCH whole culture hydrolysate

Chapter 1

Biodegradation of keratinous biomass: a waste management perspective

1.1. Introduction

Keratin-rich animal by-products such as chicken feathers, horns, and bristles are the third most abundant renewable polymeric material present in nature after cellulose and chitin (Lange et al., 2016). Keratin waste biomass is classified as a low-risk animal by-product which occurs abundantly in slaughterhouses and meat & poultry processing plants. Therefore, this type of solid residue is not suitable for human consumption and needs to be treated before its disposal into the environment (Korniłowicz-Kowalska and Bohacz, 2011; Martínez-Alvarez et al., 2015).

Keratins are highly specialized fibrous structural proteins which belong to the superfamily of scleroproteins and are synthesized inside epithelial cells of higher vertebrates and humans. The high cysteine content is the main characteristic which differentiates keratins from other structural proteins such as collagen and elastin. Cysteine residues of neighbouring polypeptide chains are covalently linked together through disulphide bridges. The resulting high degree of cross-linking brings about the formation of a tight and compact structure. The high number of disulphide bonds in the primary conformation of keratin is the main responsible for the recalcitrance of keratin-rich materials. Additionally, hydrogen bonds and hydrophobic interactions further contribute to the resistance and stability of the secondary structure of keratinous proteins. Polypeptide chains in keratin can be found in two main configurations, namely α -helix and β -sheet. The α -type is also named hard keratin, and that is because it contains a higher cysteine content which allows the formation of a larger number of disulphide bridges between cross-linking protein chains. Keratins are insoluble in water, organic solvents and weak acids and alkali and resistant to common proteolytic enzymes such as papain, trypsin and pepsin (Brandelli et al., 2010; Korniłowicz-Kowalska and Bohacz, 2011; Daroit and Brandelli, 2014).

Some bacteria, actinomycetes and keratinophilic fungi are capable of using keratin as the only source of C, N, S and energy. Specifically, they are able to synthesize microbial keratinases (EC 3.4.21/24/99.11), which are enzymes belonging to the class of serine proteases or metalloproteases, showing high levels of hydrolytic activity towards keratinous substrates (Gupta and Ramnani, 2006; Gupta et al., 2013; Daroit and Brandelli, 2014; Sharma and Devi, 2018). In particular, some keratinases are found to be members of the family of subtilisin-like serine proteases (S8 family). Nevertheless, not all subtilisin proteases are capable of degrading materials rich in keratin. The reason why a particular subtilisin shows keratinolytic activity with respect to another which does not is still unknown. Therefore, sequence homology with the most known keratinolytic protease, namely KerA from *Bacillus licheniformis* PWD-1, is usually employed to identify a potential keratinase (Lin et al., 1992).

1.2. The structure of porcine bristles

The morphological structure and chemical composition of keratins in pig bristles, also commonly known as hog hairs, make this specific type of keratinous biomass one of the most recalcitrant and hard-to-degrade among keratin-rich waste materials. For instance, the exceptional resistance of porcine bristles to chemical and enzymatic digestion largely exceeds that of poultry feathers (Łaba et al., 2015a). The morphological structure of bristles and other hair-type appendages (including wool) consists of a multi-layer cuticle and an internal cortex. Moreover, if we consider their main chemical constituents hog hairs are mostly made up of α -keratins (50–60%), matrix proteins (20–30%), and minor amounts of β -keratins (Daroit and Brandelli, 2014). In Fig.1 we can observe how single α -helix polypeptide chains are organized into coiled coil dimers which are formed by winding two filaments together and cross-linking them with disulphide bonds. Coiled coil dimers then self-assemble into an intermediate tetrameric structure made of four proteic subunits. Next, tetramers associate tail-to-head to form subfilaments called protofilaments. The protofilaments contain non-

helical N- and C- termini rich in half-cysteine residues. Fraser et al. (1962, 1988) suggested that these residues cross-link with those found in the matrix connecting the two regions through disulphide bridges. Nevertheless, they also indicated that the larger part of the disulphide linkages are found in the matrix rather than in the intermediate filaments. Lastly, protofilaments polymerize together to form the basic structural unit present inside the cortex, namely the intermediate filament (IF). The intermediate filaments are surrounded by an amorphous keratin matrix. Within the matrix mainly two types of proteins are found:

- high sulphur proteins (they contain more cysteinyl residues)
- high glycine-tyrosine proteins (they contain more glycyl residues).

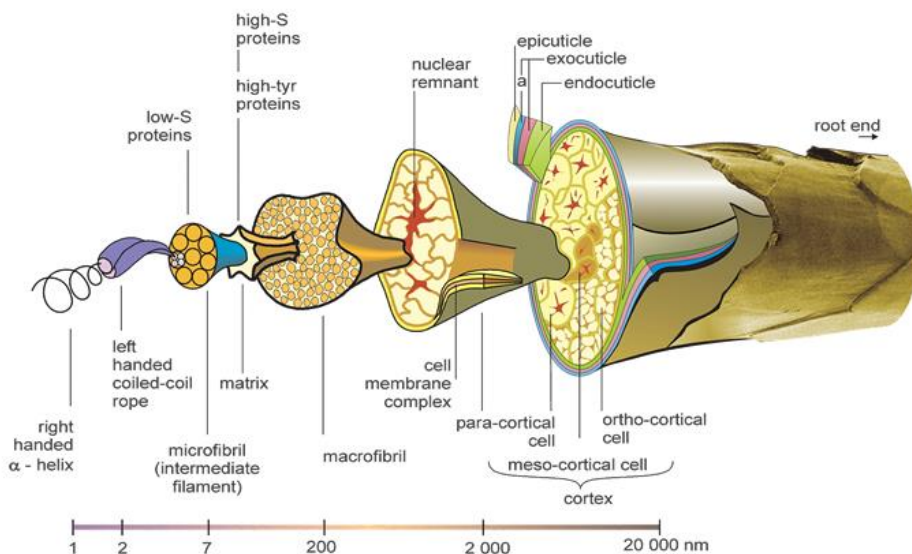


Figure1. Schematic representation of the morphology of a wool fibre according to Hill et al. (2010)

In animal hair fibres the cortex is enclosed in the cuticle, a chemically resistant structure made of flat layered cells (scales) that encircle the central core of the keratin filament. The cuticle of fine wool fibres, for instance in the case of Merino wool, consists of only one single layer of overlap-

ping scales. On the contrary, the cuticle in human hairs can be up to 10 layers thick, while in pig bristles it can contain up to 35 layers (Jones et al., 2006). Generally, the cuticle is more extensively crosslinked than the entire keratin fibre as a whole. In contrast to the cortex the crystalline domains are absent from the laminar structure of the cuticle. A thin lipid layer of 18-methyl eicosanoic acid is covalently linked to the external surface of mammalian hairs through thioester bounds (Fan et al., 2008; McKittrick et al., 2012).

The matrix is normally considered as the amorphous component of the macrofibrillar units. The IFs, instead, constitute the crystalline component of keratin and are insoluble in water. It is therefore also possible to look at hair-type keratin as a composite material where short fibres, i.e., the crystalline portion, reinforce the amorphous polymer matrix. However, if we consider its mechanical behaviour instead of its chemical structure, the matrix is more similar to a lightly cross-linked gel rather than a highly cross-linked polymer (Robbins, 2012).

1.3. Production of Microbial Keratinases

Keratins are insoluble macromolecular structures which are not capable of entering the microbial cell. Therefore, keratin-degrading microorganisms might initiate the secretion of keratin-specific proteolytic enzymes to bring about the solubilisation and breakdown of this particular protein type. Substrates with a lower molecular mass, such as oligopeptides and free amino acids, could then be transported across the cell membrane, as soon as they have been extracted and released from these keratinaceous proteins. Indeed, in most cases it was observed that extracellular keratinases were produced as inducible enzymes in mineral (basal) media containing keratinous substrates (Brandelli et al., 2010; Daroit and Brandelli, 2014). Nevertheless, in a few studies both intracellular and constitutively excreted keratinolytic enzymes have been described (Sanyal et al., 1985; Apodaca and McKerrow, 1990; Gessesse et al., 2003; Manczinger et al., 2003). In addition, it has also been demonstrated that, in some cases, non-keratinous substrates (soybean meal, casein, skim milk, soy

flour, gelatine, shrimp shell powder and brewer's spent grain among others) were capable of inducing the synthesis of keratinolytic enzymes at higher levels than those which could have been reached by utilizing keratinaceous waste materials (Wang and Shih, 1999; Mitsuiki et al., 2002; Gradišar et al., 2005; Casarin et al., 2008; Wang et al., 2008; El-Gendy, 2010; Awad et al., 2011; Rai and Mukherjee, 2011; Łaba et al., 2017). Many of the keratinases isolated from *Bacillus* and *Streptomyces* spp. have been recognized as subtilisin-like proteases (Daroit and Brandelli, 2014; Sharma and Devi, 2018). The production of proteolytic enzymes is generally associated with the microbial growth phase, and it is often observed that the peak of enzyme accumulated in the culture broth is reached at the end of the exponential growth and/or at the early stage of the stationary phase (Ramnani and Gupta, 2004; Joshi et al., 2008). As a whole, keratinase synthesis is followed by the decomposition of the keratinous substrate and the subsequent extraction of the soluble proteins, peptides and amino acids released during the hydrolysis of the keratinaceous material. However, keratinase production and keratin degradation do not occur at the same time. In fact, keratin degradation usually takes place after a certain amount of keratinolytic enzymes has been already produced (Kothari et al., 2017). Nutritional limitations, such as the lack of sufficient carbon and nitrogen sources in the cultivation medium, are generally responsible for the regulation of protease expression within the microbial cell (Contesini et al., 2018). Besides, amino acid limitations in the culture broth are normally associated with the concentration levels of guanosine triphosphate (GTP) in the microbial cell which, in turn, trigger the synthesis of proteolytic enzymes. Indeed, variations in the concentration of the nucleotide pool of the intracellular environment are usually registered when the microorganism transits between different growth stages or experiences nutritional limitations (Bierbaum et al., 1991). It has been suggested that, the extracellular production of proteases could be related to the GTP-sensing mechanism of the transcriptional pleiotropic repressor CodY (Gupta et al., 2002). Specifically, a high availability of nutrients in the extracellular environment,

which is what the microbial cell experiences during the exponential growth phase, will activate the transcriptional regulator CodY (phosphorylated form) in response to a high intracellular concentration of GTP. On the other hand, a situation in which nutrients are limited, which is what normally occurs gradually during the transition from the exponential to the stationary growth phase of the microorganism, will inactivate the CodY due to declining GTP levels inside the cell, with a resulting increase in the production of extracellular proteolytic enzymes (Sonenshein, 2005; Han et al., 2016). Therefore, it could be speculated that the synthesis of extracellular proteases could represent the evolutionary response of the microbial cell, towards the end of exponential growth phase, when trying to adapt to an extracellular environment that lacks nutrients (Daroit and Brandelli, 2014). Regulation of protease/keratinase production is associated to catabolite repression mechanisms within the microbial cell. For instance, extra addition of readily available carbon sources such as glucose, fructose, maltose and sucrose as well as rapidly metabolisable organic nitrogen sources, like those containing oligopeptides and free amino acids, inhibits the synthesis of keratinolytic and proteolytic enzymes involved in the catabolism of keratinaceous substrates (Brandelli et al., 2010). The production levels of keratinolytic enzymes achieved at the end of the manufacturing process could be dictated by the amount of microbial mass which has been accumulated inside the cultivation vessel at the end of the exponential growth phase. Therefore, the optimization of culture media composition and the search for an optimal set of cultivation parameters are of fundamental importance in order to maximize microbial growth and therefore keratinase production (Kothari et al., 2017). A possible strategy which could be implemented in order to improve the production yield of keratinolytic proteases on a specific keratin-rich waste substrate could be the addition of easily assimilable sources of carbon and nitrogen to the culture medium. By doing so, as previously mentioned, catabolite repression mechanisms would delay the production of keratinolytic enzymes. Nevertheless, after these simple sources of carbon and nitrogen have been completely consumed,

the presence of keratinous material inside the production medium, could work like an inducer and, due to the larger amount of biomass generated, a higher accumulation of keratinases could be obtained at the end of the microbial growth phase (Riffel et al., 2011; Fang et al., 2013b).

In order to render microbial keratinases commercially available cost-effective biotechnological processes for the efficient industrial-scale production of these powerful hydrolytic enzymes must be developed. Within this context, the impact of the growth media on the overall manufacturing costs associated with the industrial production of the enzymes is generally estimated to account for roughly 30-40 % of the total expenses (Joo and Chang, 2005). Therefore, keratin-rich by-products such as those derived from meat and poultry processing facilities could represent an interesting alternative as low-cost substrates for the microbiological production of keratinolytic enzymes. Feathers and feather meal are the major keratinaceous waste utilized as substrates for the biotechnological production of microbial keratinases. Although not always the case, sometimes additional carbon and nitrogen sources are also added to the culture medium. Bench-top scale production of keratinolytic enzymes is mostly investigated in batch submerged fermentation (SmF) systems (Wang and Shih, 1999; Ni et al., 2011; Zaghloul et al., 2011b; Fang et al., 2013a; Demir et al., 2015); nonetheless, up to this point, research has been mainly carried out at the shake-flask level (Sahoo et al., 2012; Yusuf et al., 2016; Lemes et al., 2016; Mazotto et al., 2017). Solid-state fermentation (SSF) techniques have also been more recently employed for producing keratin-specific proteases (Gioppo et al., 2009; Kumar et al., 2010; Paul et al., 2014a). Moreover, the possibility to obtain keratinolytic enzymes through immobilization of microbial cells on alginate-based carriers has also been explored (Prakash et al., 2010a).

When considering the physical parameters affecting production yield of keratinolytic proteases cultivation temperature, culture medium pH, type and concentration of keratin-rich waste substrate, aeration rate and agitation speed are certainly among the most important. Typical cultivation tem-

peratures during aerobic cultivation of keratinolytic microorganisms for keratinase production are in the range from 20°C to 70°C. In fact, most keratinophilic microorganisms have shown mesophilic behaviour; nevertheless a few keratin-degrading thermophilic strains have been also described in the literature (Friedrich and Antranikian, 1996; Yamamura et al., 2002a; Nam et al., 2002; Daroit and Brandelli, 2014; Sahoo et al., 2017; Wu et al., 2017). Nearly all of the experimental studies which have been described in the literature were conducted in Erlenmeyer flasks that involve the absence of pH control during microbial cultivation on keratinaceous substrate. Therefore, the majority of the reported pH values employed throughout the course of the concomitant microbial growth, keratinase production and keratin degradation, correspond to the pH of the culture broth measured when the fermentation process starts. Initial pH values are normally chosen in accordance with the type of strain employed and values ranging from slightly acidic (starting from 6.0) to alkaline (up to 10) have been reported. It has been suggested that, an alkaline pH could help to decompose keratin as it catalyses the transformation of cystine residues into lanthionine, consequently making it more accessible to the action of keratinolytic enzymes (Daroit and Brandelli, 2014; Sahoo et al., 2017). It has been described that the amount of keratinaceous substrate contained within the culture broth can strongly influence the production of keratinolytic enzymes. In particular a growing keratin-rich substrate concentration will cause the medium viscosity to increase. As a consequence, both the oxygen mass transfer rate and efficiency will decrease during cultivation of aerobic keratinolytic microorganisms, leading to oxygen transfer limitation and ending in an inadequate intracellular oxygen concentration. This will finally result in the repression of protease/keratinase synthesis. Additionally, an insufficient agitation speed can bring about the incomplete mixing of medium components and microbial cells leading to poor oxygen distribution which has a deleterious effect both in terms of enzyme yield and titre. Besides, an excessive agitation speed will result in a substantial increase in the shear stress, which in turn will damage the microbial cells causing the consequent reduction

of the overall cellular viability of the microbial culture (Suntornsuk and Suntornsuk, 2003; Zaghloul et al., 2011b; Daroit and Brandelli, 2014). Even though this may seem obvious, it is important to underline that optimal cultivation conditions for keratinase production, including media composition, vary among different microbial strains. For this reason, the first step to undertake is the establishment of the best conditions for achieving high production levels of keratinolytic enzymes. Noticeably, in a large number of studies the systematic analysis of factors influencing keratinase yield has been carried out utilising a "one-variable-at-a-time" (OVAT) methodology (Zaghloul et al., 2011a). This approach does not consider the complex set of interactions occurring among the different investigated variables and, therefore, is unable to identify a real optimum for the process parameters. A more coherent way of solving this problem involves the use of mathematical techniques based on the statistical design of experiments (DoE). Specifically, when studying a specific keratin-producing microbial strain, it is possible to employ DoE methodologies in order to describe with a mathematical relationship the interactions between input variables (medium composition and culture conditions) and output variables (keratinase production). For instance, central composite design (CCD) and response surface methodology (RSM) are among the most important and most widely employed strategies for the systematic optimization of culture media composition. With these methods, the concomitant effect of a specific set of values for the input variables on keratinase production levels can be easily tested and graphically visualized, representing an effective way to optimize cultivation conditions using a reduced number of experiments (Cai and Zheng, 2009; Fakh-fakh-Zouari et al., 2010; Tiwari and Gupta, 2010; Govarthan et al., 2015).

One of the major bottlenecks for the cost-effective production and subsequent commercialization of microbial keratinases is the low productivity and titre obtained with the wild type strains at the current state of the art in submerged fermentation (SmF). Molecular cloning and heterologous expression in a suitable recombinant host has been proposed and utilized as a possible approach to en-

hance microbial keratinase production metrics and keratin degradation efficiency (Brandelli et al., 2010; Daroit and Brandelli, 2014; Kothari et al., 2017; Sahoo et al., 2017). For instance, Lin et al., (1997) isolated the keratinase gene *kerA* from *Bacillus licheniformis* PWD-1 and employed the host *B. subtilis* DB104 that is a strain deficient in neutral and alkaline proteases, for cloning and expression of the *kerA*-codifying sequence. Specifically, they transformed the host strain with a plasmid pLB29 harbouring the keratinase gene *kerA*, the vegetative growth promoter P43 and a kanamycin resistance gene obtaining the recombinant *B. subtilis* strain FDB-29. The transformant FDB-29 was capable to express the cloned keratinase in its active form in both feather meal (FM) and Luria-Bertani (LB) media, which was in contrast to what was previously found for PWD-1, in which *kerA* synthesis was repressed when grown in LB medium. In addition, Zaghloul et al. (2011b) employed the recombinant host *B. subtilis* DB100 *his⁻ met⁻* (p5.2) to harbour a multicopy plasmid (4.7 kbp) containing the gene sequence for the expression of the heterologous alkaline protease *aprE*. The engineered feather-degrading bacterium showed an outstanding segregational and structural stability (about 100 % plasmid retention after 5 days of cultivation run) when degrading native chicken feathers in a 14 L Bio Flo 110 laboratory scale fermenter. Furthermore, in an attempt at improving the expression levels of the native *B. licheniformis* S90 keratin-specific protease *kerA* (Hu et al., 2013a) in *P. pastoris* two distinct codon optimization strategies were tested by Hu et al. (2013b). To this purpose, they cloned the keratinase gene and introduced two synonymous mutations in *kerA* by means of site-directed mutagenesis. Afterwards, they successfully expressed the two mutated genes *kerAopti1* and *kerAopti2* in *P. pastoris* X-33. The obtained recombinant *P. pastoris* pPICZaA-*kerAopti1* strain secreted the highest amount of enzyme (324 U/mL) with respect to both *P. pastoris* pPICZaA-*kerAwt*, pPICZaA-*kerAopti2* and most other reported engineered strains. Sometimes molecular cloning and heterologous expression of genes codifying keratinolytic enzymes can be used as an important tool to circumvent the potential risk posed by the presence of the whole microbial

organism because of its potential pathogenicity. In this regard, Fang et al., (2014) isolated the two keratinase-codifying genes KerSMD and KerSMF from the keratin-degrading opportunistic pathogen *Stenotrophomonas maltophilia* BBE11-1 by means of a modified TAIL-PCR method. Single genes were inserted into two pET22b vectors and effectively expressed, with the help of signal peptide pelB, extracellularly in *Escherichia coli* BL21 (DE3). Among the two obtained heterologous keratinolytic proteases, KerSMD could more efficiently decompose feather-keratin waste material when compared with the commercial keratinase kerA produced with *Bacillus licheniformis* PWD-1. Chen et al., (2015) cloned an *Aspergillus niger* *kerD* (1251 bp) keratinolytic protease gene and successfully expressed it in *Escherichia coli*. The DNA fragment codifying the mature *kerD* lacking its signal sequence was inserted into a pET30a(+) vector and transformed into the recombinant host. After being purified, the heterologous enzyme displayed maximum keratinolytic activity at 70 °C and pH 8.0 and was capable to degrade a broad range of proteinaceous substrates. The feather-degrading strain *Bacillus amyloliquefaciens* K11 fully solubilized whole feathers in no more than 24 h. The gene *kerK* (1149 bp) encoding the keratinase from *Bacillus amyloliquefaciens* K11 was cloned in a pUB110 vector and transformed into *Bacillus subtilis* SCK6 to build the recombinant plasmid pUB110-*kerK*. The plasmids harbouring the heterologous gene *kerK* were extracted from *B. subtilis* SCK6 and reintroduced into *B. amyloliquefaciens* K11. With respect to the native strain, recombinant *B. amyloliquefaciens* K11 efficiently overexpressed *kerK*, resulting in a 6-fold increase in the keratinolytic activity measured in the supernatant, and leading to the complete decomposition of the keratinous substrate in only 12 h (Yang et al., 2016). Recently, Su et al., (2017) discovered a novel alkaline surfactant-stable keratinase employing a function-driven screening-strategy based on the construction of a fosmid genomic library of the DNA material extracted from a keratin-rich soil sample. They then expressed the keratinase-encoding gene in *Escherichia coli* BL21 (DE3) and

obtained a recombinant keratinolytic enzyme which could completely degrade chicken feathers at 40 °C within 24 h.

1.4. Mechanism of keratin degradation by microbial keratinases

Kunert (1976) was the first to suggest that keratin degradation could be the result of the combined action of two main events taking place: sulphytolysis, i.e., the reduction of disulphide bridges and proteolysis, i.e., the cleavage of peptide bonds. In almost all cases, degradation of native keratin by keratinolytic proteases takes place only after cross-linking disulphide bridges have been cleaved by sulphytolysis. The cleavage of disulphide bounds modifies the structural conformation of the keratinous substrate increasing the number of available sites for the ensuing attack carried out by keratinolytic enzymes (Vignardet et al., 2001). Keratinolytic bacteria produce both disulphide reductases and keratin-specific proteases; the first type of enzyme will break down disulphide bridges of cross-linked keratins resulting in a denatured keratinous protein while the second will fragment and further decompose the concomitantly disrupted keratins releasing both peptides and amino acids. The release of sulfhydryl groups during the microbial growth of keratinophilic bacteria on keratin-rich substrate aids the reduction of disulphide bridges, making it easier to achieve complete decomposition of keratinous biomass (Daroit et al., 2009). Besides, in the presence of abiotic components, such as inorganic sulphite ions, disulphide bridges of the keratin substrate are directly cleaved to cysteine and S-sulfocysteine (Kunert, 1972a, b; Kunert, 1973; Kunert, 1976; Ruffin et al., 1976; Kunert, 1989; Grumbt et al., 2013; Sharma and Devi, 2018). Kunert (1976) reported the following scheme for the hypothesized mechanism of sulphytolysis:



Łaba et al. (2013) observed that both *Bacillus polymyxa* B20 and *Bacillus cereus* B5esz produced several sulfur compounds at different oxidation levels such as, thiols, thiosulfate, sulfite and sulfate when growing on chicken feathers as the only source of carbon and nitrogen. Nevertheless, prokar-

yotic microorganisms usually secrete minor quantities of reducing sulfur compounds. For these reasons, a different type of mechanism has been suggested in order to describe how bacteria could cleave disulfide bridges in keratinous proteins. Indeed, one of the major differences detected when comparing fungal and bacterial microbial decomposition of keratin is the elevated concentration of thiosulfates released by the eukaryotic cells in contrast to the large amount of reduced thiols generated by prokaryotic microorganisms. This accumulation of reduced thiols in the culture broth of bacteria grown on keratin-rich media could form evidence for the direct reduction of cystine bridges by some keratin-specific disulfide reductases. Disulfide reductase activity was not detected in the supernatant from a 4 days culture of *Micrococcus luteus* B1pz on chicken feather mineral basal medium. Nevertheless, 5.40 U of disulfide reductase activity was found in the cell homogenate fraction of the cultivation broth (Łaba et al., 2015a). Yamamura et al. (2002b) isolated and characterized an extracellular disulfide reductase-like protein from the new isolate *Stenotrophomonas sp.* strain D-1. They showed that the purified form of the enzyme was capable, in combination with the serine protease secreted by the same bacterium named protease D-1, to effectively degrade keratin. Moreover, Ghosh et al. (2008) detected significant disulfide reductase activity in the cell free extract when growing *Bacillus cereus* DCUW on raw chicken feather. Prakash et al. (2010b) purified and characterized two alkaline keratinases produced by *Bacillus halodurans* PPKS-2. One of the two keratin-specific enzymes showed high disulfide reductase activity and N-terminal homology with disulfide reductases together with a low keratinase activity. This enzyme was active in reducing oxidized glutathione both in presence and absence of an electron donor. Moreover, its reductase activity increased fivefold in the presence of 1 mM NADH as electron donor. A particular aspect of this enzyme was that it could show disulfide reductase activity also when no reducing agent was added to the assay mixture. Rahayu et al. (2012) found that the new isolate *Bacillus sp.* MTS from the Tangkuban Perahu crater in Indonesia produced multi-fractions of both extracellular alkaline

keratinases and disulfide reductases, and degraded whole chicken feather effectively. They actually observed that when both the purified keratinase and disulfide reductase fractions were mixed together, enzymatic activities on both feather and wool were greatly increased compared to the activity of each of the separate fractions, i.e. a synergistic effect was observed. Fang et al. (2013a) isolated and purified three keratin-specific enzymes secreted by the feather-degrading strain *Stenotrophomonas maltophilia* BBE11-1 when grown on keratin rich mineral medium. After characterizing the three keratinases they investigated the in vitro degradation of both native feather and wool by means of different combinations of the three purified enzymes. One of the three enzymes was a disulfide reductase-like protein. They demonstrated that both the keratinolytic activity and α and β keratin degradation levels could be significantly improved with the addition of the disulfide reductase to the other two enzymes. In some cases microbial colonization of keratinous substrate was a prerequisite for its effective degradation by keratinase. The reason may lie in the fact that the presence of metabolically active cells generated a suitable redox environment by constantly supplying electron donors into the medium (Bockle and Muller, 1997; Ramnani, 2005; Ramnani and Gupta, 2007; Sharma and Gupta, 2012; Gupta et al., 2013).

A large number of proteases are known for having keratinolytic activity; nonetheless, no single keratinolytic enzyme has the ability to fully decompose keratinous substrates independently. As a matter of fact, it is becoming more and more evident that in order to bring about complete degradation of keratinous materials several different keratin-specific proteases may act synergistically to break down its structure into oligopeptides and amino acids (Huang et al., 2015a; Lange et al., 2016; Sharma and Devi, 2018). When considering their catalytic mechanism of action, several keratinases secreted by *Bacillus* spp. and *Streptomyces* spp. have been identified as subtilisin-like proteases belonging to the serine proteases (S8) family. For instance, Lin et al. (1992) were the first to succeed in isolating and characterizing a keratinolytic enzyme belonging to the S8 protease fami-

ly which was obtained from the keratin-degrading bacterium *Bacillus licheniformis* PWD-1. Nevertheless, more recently, a broader variety of keratin-specific enzymes, such as, for example, keratinases which are member of the metalloproteases family, have been described (Brandelli, 2008; Brandelli et al., 2010; Brandelli et al., 2015; Lange et al., 2016; Paul et al., 2016b; Verma et al., 2017; Sharma and Devi, 2018). The microbial source, catalytic type and biochemical properties of a selected number of keratinolytic proteases was summarized in Table 1. During microbial decomposition of keratin an increase in pH is normally observed. The alkalisation of the reaction environment is the result of the release of ammonia as a consequence of deamination. Specifically, proteolytic degradation of extracted soluble proteins and oligopeptides is accompanied by a certain extent of amino group removal of their amino acidic component. Deamination generates an alkaline environment that is optimal for the catalytic action of alkaline proteases.

1.5. Keratinaceous by-products: waste management

Only in 2014, approximately 222,000 tons of wet pig bristles were generated as a waste inside the European Union. The cost for the disposal of this biological waste was evaluated at around 0.02 €/kg (Gachango et al., 2017). The effective reutilization of such a type of keratinous by-product constitutes an unsolved problem due to its outstanding resistance to chemical and enzymatic digestion (Łaba et al., 2015b). Within the rendering industry hydrothermal treatment is the most widely applied method to process keratinous waste biomass (Papadopoulos et al., 1985). Clean, undecomposed chicken feathers or porcine bristles are steam pressure-cooked and a more digestible hydrolysed keratin meal is obtained (Hertrampf and Piedad-Pascual, 2000a, 2000b). It is possible to produce hydrolysed keratin meal both in batch cookers and in high-volume continuous hydrolysers. This is actually not a real hydrolysis process but more correctly a denaturation process in which molecular constituents of the keratin structure are split while the properties of proteins remain unaltered. Hydrothermal treatment for the production of keratin meal involves three major steps, i.e.: 1)

hydrolysis, 2) drying, and 3) grinding. During the hydrolysis step, the keratin waste is steam cooked in an autoclave employing an optimum combination of temperature (~ 130 - 145 °C), pressure (~ 207 - 414 kPa), and time (~ 20 -150 min) in order to hydrolyse the keratinaceous proteins (McCasland and Richardson, 1966; Latshaw et al., 1994). Hydrolysis is normally followed by a drying step, during which the amount of water contained in the keratin hydrolysate is reduced to a desired level. Lastly, the dried material is ground by hammer milling to obtain a powder with an average particle size suitable for its inclusion in animal feeds. However, steam pressure-cooking of native feathers and hog hairs is an expensive process route to obtain both types of keratin protein hydrolysate, also considering the significant amounts of energy consumed during the treatment. Additionally, when hydrothermal methods are employed some essential amino acids such as cysteine, methionine and lysine are lost and nonproteinogenic and therefore non-nutritive amino acids are formed, which is the case of lanthionine and lysinoalanine (Latshaw et al., 1994; Chojnacka et al., 2011). As a consequence, the final product is characterised by poor digestibility and low nutritional value, which considerably limits its use as ingredient for animal feed (Bertsch and Coello, 2005; Mazotto et al., 2017). Moreover, the great batch-to-batch variability in nutrient composition and bioavailability form has a considerable effect on the protein quality of commercial feather and hair meals and is one of the most relevant issues regarding their inclusion in feed formulations (Wang and Parsons, 1997; Grazziotin et al., 2008). In particular, according to Hertrampf and Piedad-Pascual (2000b) the use of porcine bristle meal as a protein source for aquaculture diets is not advisable. Nonetheless, they strongly recommended supplementing with certain amino acids if pig hair meal has to be employed as a partial replacement for high quality protein in aquaculture feed. In the last three decades, the development of microbiologically mediated methods for the valorisation of slaughterhouse keratin-rich by-products such as poultry feathers and hog hairs for production of protein-enriched feed for animal nutrition has gained significant momentum. Microbiologi-

cal conversion of keratin-rich by-products can be considered an attractive way of transforming this particular type of recalcitrant waste material into products of commercial interest (Korniłowicz-Kowalska and Bohacz, 2011; Verma et al., 2017). As a matter of fact, protein hydrolysates obtained from the biodegradation of keratin-rich animal processing by-products by microbial keratinolysis show enhanced digestibility and nutritional quality with respect to those obtained through hydrothermal processes. In particular, bacterial-treated feathers may possess similar nutritional properties when compared to soybean meal (Williams et al., 1991). For instance, the establishment of microbiological processes for the hydrolysis of these keratinous waste materials under industrially implementable production conditions to obtain high protein-feed ingredients could replace a significant fraction of the fish meal used in aquaculture feed formulation.

The aquaculture industry is one of the fastest growing sectors in food production, and it already accounts for approximately half of the seafood consumed in the world. Fish meal constitutes one of the main ingredients of fish feed and represents about 40% of its total weight. The soluble proteins, peptides and free amino acids extracted during the enzymatic degradation of keratin will supply an economical and risk-free source of high protein-feed ingredients which can be used to replace a significant fraction of the fish meal used in aquaculture feed formulation. Novel technologies for making proteins for feeding farmed fish are gaining significant importance. Their goal is to reduce the use of wild fish in aquaculture which is considered an unsustainable practice. Currently, about one quarter of the wild fish caught each year is used as food for cultivating aquatic species. Within this context, the microbiological processing of keratin waste biomass can address this problem by solving it with a holistic approach which integrates both waste remediation and resource recovery, which is completely in line with the current focus on circular economy concepts.

Graziottin et al. (2007) obtained protein-rich hydrolysates through microbial degradation of native chicken feathers employing the bacterium *Vibrio sp.* strain kr2. They proved that the *in vitro* digest-

ibility of the proteins from the whole culture hydrolysate (WCH) was lower than soybean meal but higher with respect to feather meal and hammer milled raw chicken feathers. On the other hand the protein-rich feather hydrolysate obtained using only the supernatant (CSH) showed a similar *in vitro* digestibility when compared to soy protein meal (Grazziotin et al., 2006). Additionally, they evaluated some *in silico* nutritional parameters for both the WCH and CSH and for the untreated feather meal as well. The best results both in terms of protein efficiency ratio (PER) and biological value (BV) were attained for the WCH while the digestibility-corrected amino acid score (PDCAAS) was superior in the case of the CSH. Tiwary and Gupta (2010) isolated and characterized a thiol activated dimeric serine protease from *Bacillus licheniformis* ER-15 that was able to degrade native feather keratin. In particular, the purified keratinolytic enzyme alone was capable of fully decomposing 3 % (w/v) chicken feathers in 12 h including the dissolution of the shaft. Remarkably, no reducing agent was required in order to achieve complete keratinaceous substrate degradation. The obtained feather meal contained all essential amino acids, including 14 g of nitrogen and 87 g of crude proteins per 100 g of dry weight, and an in-vitro digestibility of 73.4 % (Tiwary and Gupta, 2012). Stiborova et al. (2016) compared the nutritional value and digestibility of protein hydrolysates prepared through biological decomposition of raw chicken feathers employing three different methods: microbial degradation of the raw feathers by the keratinolytic bacterium *Pseudomonas* sp. P5, enzymatic hydrolysis with semi-purified keratinases previously produced by the same microbial strain and alkaline chemical hydrolysis under mild conditions. In the first case the protein hydrolysate included about 301 mg L⁻¹ and 6.2 g L⁻¹ of free amino acids and peptides respectively. With the second approach, while the level of released free amino acids was considerably higher (~ 1191 mg L⁻¹), only 3.3 g L⁻¹ of peptides were produced. Finally, regarding the third method a very significant increase in released peptides was observed (17.2 g L⁻¹) but, on the other hand, a very low amount of free amino acids was extracted. Afterwards, each of the three obtained feather

protein hydrolysates was tested regarding its potential as peptone replacement in a standard culture growth for *E. coli* cultivation as a test organism. The microorganism was shown to be able to grow with each of the three nutrient sources; nevertheless it was in the case of the alkali treated keratin that the highest amount of microbial mass was accumulated.

1.6. Antioxidant properties of keratin protein hydrolysates

A considerable fraction of the fish meal included in fish feed formulation is normally based on marine ingredients, resulting in a large content of polyunsaturated n-3 fatty acids and thus a high vulnerability towards lipid oxidation. Nowadays, synthetic antioxidants, such as ethoxyquin (EQ) and butylated hydroxytoluene (BHT), are added to fish meal in order to avoid the occurrence of this phenomenon. A considerable fraction of these antioxidants is transferred to the fish fillet and despite the EU regulation imposes two weeks of starvation before the farmed fish can be slaughtered, this is normally not sufficient to get rid of these synthetic compounds from the fillet. Concerns regarding the use of synthetic antioxidants have prompted the authorities to reduce maximal residual levels of antioxidants allowed in food for human consumption to levels which are just slightly above those which are usually detected inside seafood produced by the aquaculture industry. For that reason, the inclusion of feed ingredients containing natural antioxidants in fish feed formulations would be advantageous both for the aquaculture sector and for the consumer in respect of its health and well-being (Hamre et al., 2010). Interestingly, a few studies have found that some protein hydrolysates obtained through biological digestion of keratinous by-products displayed remarkable antioxidant properties. For instance, both feather and wool protein hydrolysates prepared through microbial degradation of native chicken feathers and sheep wool waste respectively using the keratinolytic bacterium *Bacillus pumilus* A1 had interesting antioxidant power when tested for their DPPH radical-scavenging activity and Iron (III) reducing power (Fakhfakh et al., 2011; Fakhfakh et al., 2013). Moreover, the maximum extraction levels of peptides and free amino acids were

obtained for both keratin-rich substrates ($\sim 42.4 \text{ g L}^{-1}$ for chicken feather and $\sim 39.7 \text{ g L}^{-1}$ for sheep wool) when growing *B. pumilus* A1 in a medium containing 50 g L^{-1} of waste keratin and with an initial pH of 10.0, for 48 hours at 45°C . The resulting feather and wool protein hydrolysates had a very high in vitro digestibility (98% and 97% respectively). Fontoura et al. (2014) investigated the antioxidant and antihypertensive properties of protein hydrolysates produced by the keratin-degrading bacterium *Chryseobacterium* sp. kr6 when grown on a mineral medium containing thermally denatured chicken feathers. During their study, they found that the largest concentration of released soluble proteins (about 27.2 g L^{-1}) was measured after 4 days of cultivation in keratin medium with 75 g L^{-1} pretreated feathers. Next, they tested both DPPH and ABTS scavenging activities on the obtained supernatants. Maximum levels of DPPH antioxidant activities ($\sim 83\%$) in feather protein hydrolysates were observed after 48 hours of cultivation, with a culture medium pH in between 8 and 9 and initial keratinous substrate concentrations of 25 and 50 g L^{-1} . Additionally, ABTS scavenging activity of protein hydrolysates also peaked after 48 hours (up to 90%) and showed similar values for concentrations of feather in the culture medium from 25 up to 75 g L^{-1} . Finally, the feather protein hydrolysate exhibited the capability to inhibit both angiotensin-I converting enzyme (ACE) and dipeptidyl peptidase-IV (DDP-IV) activities by 65% and 44% respectively when tested on the extracted crude protein hydrolysates. In a different study, Wan et al., (2016) isolated and identified a new antioxidant peptide, with the sequence Ser-Asn-Leu-Cys-Arg-Pro-Cys-Gly, from a protein hydrolysate obtained after digestion of chicken feathers with the keratin-degrading microorganism *Bacillus subtilis* S1-4. Lemes et al. (2016) investigated the use of several agro-industrial by-products (i.e., feather meal, reused feather meal, residual microbial mass, 50% feather meal + 50% residual microbial mass) for the production of both proteolytic enzymes and antioxidant compounds by means of *Bacillus* sp. 45 cultivations on such residual substrates. During the course of their study it was found that the highest ABST antioxidant activity was ob-

served for the feather meal hydrolysate ($53.9 \text{ mg}_{\text{TE}} \text{ g}^{-1}$), while the largest reducing power measured with the FRAP method was detected in the case of the hydrolysate obtained from the microbial digestion of the residual biomass. Lastly, the samples prepared from the mixed substrate were those showing the highest DPPH scavenging power. Given these facts, it is fair to assume that the described keratin protein hydrolysates could represent a natural source of bioactive compounds to be included in feed formulations and eventually also food and pharmaceutical products (Sinkiewicz et al., 2018).

1.7. Motivations and project goals

In this PhD thesis, we have focused our attention towards a particularly hard-to-degrade keratin-rich by-product, namely pig bristles (which is also known as hog hairs). As a matter of fact, the conventional thermo/chemical methods employed for processing porcine bristles into a more digestible dietary protein result in a product which is usually deficient in sufficient essential amino acids and still characterized by a considerably low digestibility. Therefore, aiming at converting this special type of keratinous waste biomass into nutritionally valuable keratin protein hydrolysates, we have decided to direct our research effort towards the investigation of the microbial route to bring about the effective decomposition of pig bristles.

Therefore the main objectives of this PhD thesis are:

- to investigate the ability of the keratinolytic filamentous bacterium *Amycolatopsis keratiniphila* D2 in directing the efficient degradation of thermally pretreated pig bristles;
- to develop a biotechnological process that can accomplish the cost-effective microbial conversion of porcine bristles into a nutritionally valuable protein-rich keratin hydrolysates which could then be included in fish feed formulations as an alternative protein source for aquaculture.

In detail, in Chapter 2 the statistical optimization of a single-stage microbial degradation process employing *A. keratiniphila* D2 was presented. Moreover, in Chapter 3 we demonstrated that with a

novel two-stage process strategy, operated at high solids loadings, production metrics could be significantly improved with respect to the case illustrated in Chapter 2. On the other hand, Chapter 4 explored two different strategies both employing cell-free crude keratinase extracts produced by *A. keratiniphila* D2 to bring about the keratinolytic hydrolysis of porcine bristles at high solids loadings. The nutritional quality of the obtained protein hydrolysates was also evaluated. Finally, Chapter 5 dealt with the investigation and following characterization of some of the keratin-specific proteolytic enzymes found in culture supernatants when growing *A. keratiniphila* D2 on keratin-rich substrates.

Table 1 Some of the characterized keratinolytic proteases from keratinolytic microorganisms

Microorganism	Catalytic Type	Mol. Wt. (kDa)	Optimum pH	Optimum T (°C)	Reference
<i>Actinomadura keratinilytica</i> Cpt29	Serine	29.2	10	70	Habbeche et al. (2014)
<i>Aspergillus oryzae</i>	Metallo	60	8	50	Farag and Hassan (2004)
<i>Bacillus altitudinis</i> RBDV1	Serine	43	8	85	Pawar et al. (2018)
<i>Bacillus cereus</i> DCUW	Serine	80	8.5	50	Ghosh et al. (2008)
<i>Bacillus circulans</i> DZ100	Serine	32	12.5	85	Benkiar et al. (2013)
<i>Bacillus licheniformis</i> PWD-1	Serine	33	7.5	50	Lin et al. (1992)
<i>Bacillus licheniformis</i> K-508	Thiol	42	8.5	52	Rozs et al. (2002)
<i>Bacillus licheniformis</i> ER-15	Serine	58	11	70	Tiwary and Gupta (2010)
<i>Bacillus pumilus</i>	Serine	65	8	65	Kumar et al. (2008)
<i>Bacillus pumilus</i> NRC21	Serine-metallo	30	7.5/8.5	45/50	Tork et al. (2016)
<i>Bacillus subtilis</i> RM-01	Serine	20.1	9	45	Rai et al. (2009)
<i>Caldicoprobacter algeriensis</i> TH7C1 ^T	Serine	33.2	7	50	Bouacem et al. (2016)
<i>Chryseobacterium</i> sp. kr6	Metallo	64	8.5	50	Riffel et al. (2007)
<i>Fervidobacterium pennavorans</i>	Serine	130	10	80	Friedrich and Antranikian (1996)
<i>Meiothermus taiwanensis</i> WR-220	Serine	28	10	65	Wu et al. (2017)
<i>Microbacterium</i> sp. kr10	Metallo	42	7.5	50	Thys and Brandelli (2006)
<i>Myrothecium verrucaria</i>	Serine	22	8.3	37	Moreira-Gasparin et al. (2009)
<i>Nocardiopsis</i> sp. TOA-1	Serine	20	>12.5	60	Mitsuiki et al. (2004)
<i>Nesterkonkia</i> sp. AL-20	Serine	23	10	70	Gessesse et al. (2003)
<i>Streptomyces</i> sp. S7	Serine-metallo	44	11	45	Tatineni et al. (2008)
<i>Streptomyces</i> sp. AB1	Serine	29.9	11.5	75	Jaouadi et al. (2010)
<i>Thermoanaerobacter keratinophilus</i>	Serine	135	8	85	Riessen and Antranikian (2001)
<i>Trichophyton</i> sp. HA-2	Serine	34	7.8	40	Anbu et al. (2008)

Chapter 2

An experimental design for the optimal biodegradation of pretreated pig bristles by the keratinolytic actinomycetes *Amycolatopsis keratiniphila* D2.

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Abstract

The effect of culture conditions on bristle protein hydrolysate (BPH) production during microbial degradation of porcine bristle meal (PBM) by the keratinolytic bacterium *Amycolatopsis keratiniphila* D2 was investigated. To begin with, a Plackett-Burman factorial design was used to assess the significance of the impact that selected factors had on the levels of total proteins (i.e., crude soluble proteins + NH₂-free amino groups) extracted. The concentration of the keratinous substrate and the age of the inoculum were found to be the most influential independent variables. Afterwards, a Box-Behnken experimental design was employed to predict the optimal levels for the four most crucial fermentation parameters. The maximum concentration of total extracted proteins was predicted for a bacterial degradation process carried out at a cultivation temperature of 35 °C, an initial pH of 6.9, a keratinous substrate concentration equal to 7% (w/w) and utilizing a seed culture that had aged for 48 hours. Finally, submerged cultivation experiments in a 3-L aerobic fermenter, which resulted in approximately 18.5 g/L of hydrolysed proteins, confirmed the model predictions obtained during the shake flask trials. The bioconversion process described in this work represents a valid biotechnological alternative for the effective management of this hard-to-degrade hair-type keratinous waste stream.

Abbreviations

PBM porcine bristle meal

BPH bristle protein hydrolysate

RSM response surface methodology

1. Introduction

Keratins are fibrous structural proteins which are insoluble in water and recalcitrant to enzymatic lysis with common proteolytic enzymes (Kornilłowicz-Kowalska and Bohacz, 2011; Daroit and Brandelli, 2014). The main element in the structure of keratin consists of a supercoiled protein motif of α -helices (α -keratins, 40-60 kDa) or β -sheets (β -keratins, 10 kDa) that is organized in a rigid secondary structure stabilized by the presence of a high number of disulphide linkages, hydrogen bonds and hydrophobic interactions (Kothari et al., 2017). Keratinous proteins are generally found in the epidermis and epidermal appendages of vertebrates such as, scales, feathers, beaks, horns, hooves, bristles and wool. Indeed, keratinaceous biomass as a whole constitutes, after cellulose and chitin, the third most abundant biopolymer present in nature (Lange et al., 2016). Among others, bristles generated in slaughterhouses and meat processing plants constitute a particularly underestimated and thus underexploited category of keratinous by-product and a serious environmental problem as well. As a matter of fact, the effective management of this hair-type keratinous waste is hindered by its exceptional sturdiness and outstanding chemical resistance (Fakhfakh et al., 2013; Fang et al., 2013b; Łaba et al., 2015a; Łaba et al., 2017). In 2014 about 250 million pigs were reared in the EU alone (EUROSTAT, 2016); taking into account that more than 80% of the porcine bristles dry weight can consist of proteins, one can estimate that approximately 60 k-tons of dry proteins could be recovered from this particular type of residual biomass which in turn could be recycled into a renewable protein feed for animals. Nevertheless, conventional methods such as alkali

hydrolysis or steam pressure cooking (Latshaw et al., 1994; Chojnacka et al., 2011) normally employed to convert pig bristles into a more digestible dietary protein generate a porcine bristle meal (PBM) that is usually lacking sufficient essential amino acids (mainly methionine, histidine, tryptophan and lysine) and has still a relatively low digestibility (Hertrampf and Piedad-Pascual, 2000b; Bertsch and Coello, 2005). On the other hand, microbiological degradation of keratinous by-products can represent a valid alternative to these traditional methods. Indeed, some bacteria, actinomycetes and keratinophilic fungi, during millions of years of evolution, have developed the capability to utilize keratin as their only source of C, N, S and energy. That is because they are able to synthesize proteolytic enzymes known as keratinases that can cleave peptide linkages within keratin polypeptide chains and therefore decompose keratin-rich waste materials (Verma et al., 2017). For instance, feather protein hydrolysates obtained through microbial degradation of native chicken feathers by the bacteria *B. licheniformis* PWD-1 and *Vibrio* sp. strain kr2 showed similar nutritional characteristics when compared to soybean meal (Williams et al., 1991; Grazziotin et al., 2006).

The objective of this study was to optimize the microbiological conversion of pretreated porcine bristles into keratin protein hydrolysate by the keratinolytic filamentous bacterium *Amycolatopsis keratiniphila* D2. To this end a Plackett-Burman screening design followed by a response surface method (i.e. a Box-Behnken experimental design) were employed to select and then optimize the cultivation parameters which exerted the largest effect on the extent of keratin hydrolysis. Finally, the validity of the obtained set of optimal conditions for keratin hydrolysis was put to the test by means of bench-scale submerged fermentation experiments.

2. Materials and Methods

2.1. Porcine bristle meal

The slaughterhouse by-product used as C, N and energy source for microbial growth and employed as well to prepare azokeratin for activity assays was kindly supplied by DAKA SARIA Group A/S

(Løsning, Denmark). The keratinous residual material consisted mainly of porcine bristles, which were chopped, thermally pretreated (150 °C, 6 bars, 20 min), dried and finally crushed into smaller particles.

2.2. Chemicals and gases

All chemicals employed in this study were of analytical grade and were purchased from Sigma Aldrich ApS (Brøndby, Denmark); gases were supplied by AGA A/S (Copenhagen, Denmark).

2.3. Microorganism and media composition

The strain *Amycolatopsis keratiniphila* D2 (DSM 44409) was obtained from DSMZ (Braunschweig, Germany). Seed culture glycerol stocks were prepared and stored at -80 °C prior to use. *A. keratiniphila* D2 was cultivated in mineral keratin (Basal) medium formulated according to the following composition (g/L): 0.75 NaCl, 1.75 K₂HPO₄, 0.25 MgSO₄·7H₂O, 0.055 CaCl₂, 0.010 FeSO₄·7H₂O, 0.005 ZnSO₄·7H₂O, 10.53 (1 % w/w) PBM powder. The medium was sterilized at 121 °C for 20 min before use.

2.4. Inocula preparation

Inocula were prepared by propagating frozen microbial cell stocks of *A. keratiniphila* D2 in Erlenmeyer flasks (500 mL) containing 100 mL of GYM medium (4.0 g/L glucose, 4.0 g/L yeast extract, 10.0 g/L malt extract); the initial pH of the culture broth was adjusted to 7.2 before autoclaving. Each flask was inoculated with 1 mL of a glycerol stock (20 % v/v) of bacterial cells and incubated on an orbital shaker (200 rpm) at 28 °C for 48 hours. Microbial cells were harvested by centrifugation (4000 rpm for 20 min at 4°C), washed twice in sterile phosphate buffer saline (PBS) solution (15 mM NaCl, pH 7.2) and again resuspended in an appropriate volume of PBS solution.

2.5.Keratinase assay

Crude keratinase activity was assayed on supernatant obtained after centrifugation ($12000 \times g$, 5 min) of the fermentation broth with azokeratin as a substrate by the following method. The reaction mixture contained 200 μ L of enzyme preparation and 1400 μ L of 10 g/L azokeratin in 50 mM Tris buffer, pH 8.0. The mixture was incubated for 15 min at 50 °C; the reaction was stopped by the addition of 1600 μ L Trichloroacetic acid (TCA) to a final concentration of 100 g/l. After centrifugation at $10,000 \times g$ for 5 min, the absorbance of the supernatant was determined at 440 nm. One unit of enzyme activity was the amount of enzyme that caused a change of absorbance of 0.01 at 440 nm after 15 min incubation at 50 °C.

2.6.Crude soluble proteins and NH₂-free amino acids determination

Soluble protein concentration was determined by the bicinchoninic acid (BCA) assay (Smith et al., 1985) employing bovine serum albumin (BSA) as protein standard (Pierce™ BCA Protein Assay Kit, Thermo Scientific). The concentration of NH₂-free amino groups was measured by the ninhydrin method as described earlier by Pearce et al. (1988), with leucine as a standard.

2.7.Optimization of pretreated porcine bristles microbial degradation

Microbial degradation of pretreated pig bristles by *A. keratiniphila* D2 was optimized by following a three-step approach: (Step1) identification of the most significant parameters (media components and cultivation variables) influencing the response of the biological system; (Step2) selection of the inoculum volume; (Step3) optimization of the two most significant factors identified at the end of step 1 together with the cultivation temperature and the initial pH of the culture broth (four factors in total). The sum of the crude soluble proteins and NH₂-free amino acids, here called the “total extracted proteins”, released during the microbiological decomposition of the porcine bristle meal, was used to represent the dependent variable that was employed to quantify the extent of degradation of the keratinous substrate. Each value obtained for the dependent variable corresponded to the

maximum concentration of total extracted proteins reached after 5 days of cultivation. All cultures were carried out in duplicate in Erlenmeyer flasks (250 mL) containing 50 mL of keratin (Basal) medium.

(Step1) Preliminary screening of significant factors influencing the levels of total extracted proteins was carried out by means of a Plackett-Burman factorial design. The effect of 10 independent variables, namely PBM concentration, inoculum age, inoculum size, NaCl, KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, CaCl_2 , peptone, NH_4Cl and urea, was studied at two different levels; the minimum and the maximum levels for each of the investigated parameters are shown in Table 1. JMP 13.0 software (SAS institute Inc.) was used to generate a set of 12 experiments. Each experiment was executed in duplicate. The effect of the independent variables on the total extracted proteins was ranked by calculating the unpaired two sample Student t-test and the P-value for each of the factors was analysed.

(Step2) The effect of the inoculum size on total extracted proteins was evaluated in 250 mL Erlenmeyer flasks, each one containing 50 mL of mineral medium with 20 g/L PBM, initial pH of 7.5, 28°C, 200 rpm and 2, 6, 10, 14, 17.5 and 20% (v/v) inoculum volume. Sampling was conducted after 24, 72 and 120 hours of incubation. Each unit (i.e. 1%) of inoculum contained about 2.6 mg of dry microbial cells ($\sim 3.95 \text{ OD}_{600}$). Each experiment was conducted in triplicate.

(Step3) Statistical optimization of the four most influential parameters namely PBM concentration (A), inoculum age (B), temperature (C) and initial pH (D) was conducted by means of a 27-run Box-Behnken design with three replicates at the centre point. Each experiment was executed in duplicate. The relationship among the investigated factors was described by means of the following second order polynomial equation:

$$Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_4D + \beta_5AB + \beta_6AC + \beta_7AD + \beta_8BC + \beta_9BD + \beta_{10}CD + \beta_{11}AA + \beta_{12}BB + \beta_{13}CC + \beta_{14}DD$$

where Y was the predicted response and β_i were fixed regression coefficients. The experimental design, the polynomial regression, the model response, i.e., predicted concentration of total extracted proteins, the optimal values for the four dependent variables, the coefficient of determination (R^2), the analysis of variance (ANOVA) and 3D-plots were calculated with JMP 13.0 software (SAS institute Inc.).

2.8. Microbial hydrolysis of pretreated pig bristles in a laboratory scale fermenter

Batch submerged cultivations of the filamentous bacterium *A. keratiniphila* D2 were carried out in two identical 3-L bioreactors (Applikon, Delft, The Netherlands), with a 2.0-L working volume. Both fermenters were equipped with two 6-bladed Rushton impellers. Cultivation parameters were optimized in order to maximize both the rate of keratin degradation and the extent of crude protein extraction and solubilisation as well: the initial pH of the keratin basal medium containing 7 % (w/w) bristle meal powder was adjusted to a starting value of 6.9; the temperature was set at 35 °C; fully aerobic conditions (dissolved oxygen concentration ≥ 30 %) were ensured by flowing 1 vvm of air through the vessel; stirring was kept at a constant rate of 500 rpm. Fermenters were inoculated with 10% (v/v) of fresh inoculum (≈ 0.25 mg cdw/ml) and run under the previously described condition for up to 120 h. During the course of the cultivation of *A. keratiniphila* D2 in a keratinous waste containing medium an increase of pH was observed. Once a pH value of 8.0 was reached, in order to avoid a further increase of alkalinity in the culture broth, the pH was stabilized around a set point value of 8.0 and then controlled by adding 2M H_2SO_4 with a peristaltic pump. Lastly, microbial mass concentrations were determined according to the method developed by Zhao et al. (2013) (see Supporting Figure S1).

2.9. Statistical analysis

Experimental data were analysed for statistical significance ($p < 0.05$) using one-way ANOVA, followed by Tukey's honestly significantly different (HSD) test.

3. Results and discussion

3.1. Screening of medium components and cultivation parameters with Plackett-Burman design

A total of ten independent variables, viz. PBM concentration, inoculum age, inoculum size, NaCl, KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, CaCl_2 , peptone, NH_4Cl and urea, were submitted to a screening experiment designed by means of the Plackett-Burman method, aiming at selecting the parameters which influenced the most the overall yield of total extracted proteins (Table 1). Among the investigated parameters, PBM concentration, inoculum age, KH_2PO_4 , size of inoculum, NaCl and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, were found to have a positive effect on the dependent variable while NH_4Cl , urea, CaCl_2 and peptone were discovered to exert a negative influence on the amount of proteins released (Table 2). Nevertheless, from the statistical analysis of the data which were summarized in Table 2, it was revealed that only four variables, namely PBM concentration, inoculum age, NH_4Cl , and urea affected significantly the level of total extracted proteins during the degradation process. Indeed, it was previously reported by several authors that the keratinous substrate was the main factor influencing both protein extraction and keratinases production by different types of keratinolytic microorganism (Embaby et al., 2010; Paul et al., 2014; Demir et al., 2015; Łaba et al., 2018). This finding should not be considered as a surprise since the keratinaceous waste material normally constitutes the main source of C, N and energy for the keratin-degrading strain. In general, the addition of a supplementary source of nitrogen to the culture medium can generate very different results regarding keratinase secretion and keratin hydrolysate production and, thus, should be evaluated on a case-by-case basis (Brandelli et al., 2010). For instance, for some microbial species it was described that supplementing the culture broth with peptone, tryptone, yeast extract, urea and NH_4Cl (Graziotin et al., 2007; Cai and Zheng, 2009; Riffel et al., 2011) had a positive influence on keratinase and protein hydrolysate production while in the case of other types of microorganism the addition to

the fermentation medium of casein, soy bean protein, gelatine, alanine, urea, NH_4NO_3 and NH_4Cl (El-Refai et al., 2005; Da Gioppo et al., 2009; Fakhfakh-Zouari, et al., 2010; Riffel et al., 2011), displayed a negative impact on keratinolytic enzymes secretion and the percentage of keratinous substrate degradation. When considering *A. keratiniphila* D2, all the three tested extra nitrogen sources affected the final amount of total extracted proteins negatively. The occurrence of this phenomenon could probably be related to a catabolite repression mechanism of the keratinase synthesis (Daroit and Brandelli, 2014). Therefore, it was chosen to reformulate the medium composition by removing NH_4Cl , urea and peptone from its components, and it was decided to further investigate the effect of the pretreated bristles concentration and of the age of the seed culture on the protein extraction levels.

3.2.Effect of inoculum size on degradation of pretreated porcine bristles

In spite of the fact that the Plackett-Burman design did not rank the inoculum size among the factors which were exerting the most significant influence on the total amount of extracted proteins, it was in any case decided to investigate how the inoculum volume could act on the dependent variable and therefore affect the performance of *A. keratiniphila* D2 in degrading keratin. Indeed, the size of the seed culture is often statistically identified as one of the factors entering an optimization campaign involving the use of response surface methodology (RSM) design of experiment. For instance, Embaby et al. (2010) revealed that the inoculum size did not show any relevant effect on the yield of crude soluble proteins and NH_2 -free amino acids obtained during microbial degradation of both chicken feathers and sheep wool with a recombinant *B. subtilis* strain. Nevertheless, Cai and Zheng (2009) reported that the maximum inoculum size which was tested in their one variable at a time (OVAT) optimization, i.e. 10% (v/v), was optimal for production of keratinolytic enzymes by *Bacillus subtilis* KD-N2 in human hair mineral medium while lower volumes of microbial cells resulted in lower keratinase yields. In the current experimental work, we studied the influence ex-

erted by the inoculum volume on the total amount of crude proteins extracted and thus recovered into the culture medium during the microbial decomposition of the pretreated porcine bristles. The data reported in Figure 1 illustrate how the yield of total extracted protein varied as a function of the cultivation time for increasing seed culture volumes ranging from a minimum of 2% (v/v) up to a maximum of 20% (v/v). As a result, it was clearly shown that after five days of cultivation, approximately the same concentration of extracted crude soluble proteins (4.66 ± 0.07 mg/mL) was reached in each one of the six experimental runs. Therefore, it was concluded that an intermediate inoculum volume of 10% (v/v) could be suitably employed in the following part of the study.

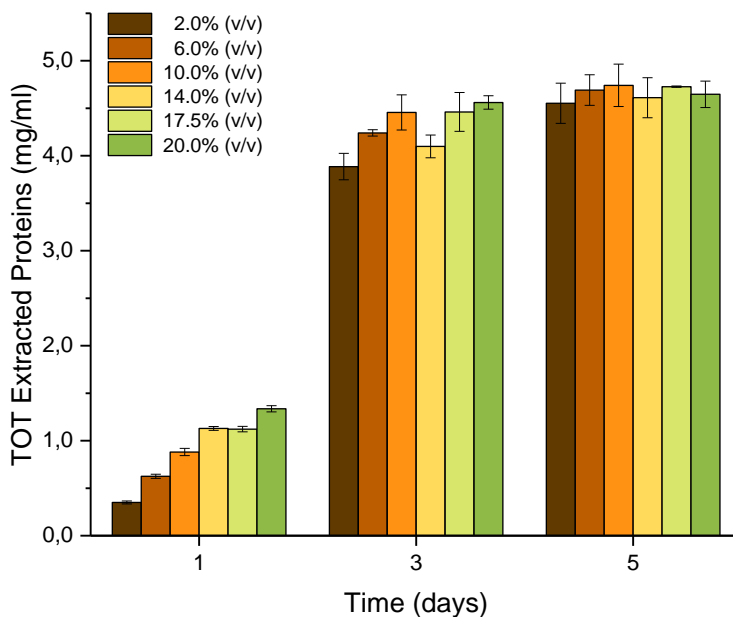


Fig. 1. Influence of the size of inoculum on the total amount of extracted crude proteins by *A. keratiniphila* D2. Cultivations were performed in mineral medium with 20 g/L PBM, initial pH of 7.5, 28°C and 200 rpm. Sampling was conducted at 24, 72 and 120 hours. Each unit of inoculum (i.e. 1%) contained about 2.6 mg of dry microbial cells (~ 3.95 OD₆₀₀).

Tab. 1. Plackett-Burman screening design for the investigated factors and observed responses

Run	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	X ₈	X ₉	X ₁₀	Total Extracted Proteins (mg/mL)
1	78.65	24	10	0.25	0.05	0.05	0.1	10	10	0	3.75 ± 0.07
2	78.65	48	2	1.5	0.05	0.05	0.01	10	10	10	3.53 ± 0.09
3	10.53	48	10	0.25	3	0.05	0.01	0	10	10	1.39 ± 0.01
4	78.65	24	10	1.5	0.05	0.5	0.01	0	0	10	8.42 ± 0.21
5	78.65	48	2	1.5	3	0.05	0.1	0	0	0	15.17 ± 0.58
6	78.65	48	10	0.25	3	0.5	0.01	10	0	0	15.39 ± 1.12
7	10.53	48	10	1.5	0.05	0.5	0.1	0	10	0	1.82 ± 0.01
8	10.53	24	10	1.5	3	0.05	0.1	10	0	10	1.46 ± 0.03
9	10.53	24	2	1.5	3	0.5	0.01	10	10	0	1.10 ± 0.12
10	78.65	24	2	0.25	3	0.5	0.1	0	10	10	2.30 ± 0.06
11	10.53	48	2	0.25	0.05	0.5	0.1	10	0	10	1.16 ± 0.02
12	10.53	24	2	0.25	0.05	0.05	0.01	0	0	0	2.41 ± 0.01

X₁: PBM concentration (mg/mL); X₂: Inoculum age (h); X₃: Inoculum size (% v/v); X₄: NaCl (mg/mL); X₅: KH₂PO₄ (mg/mL); X₆: MgSO₄·7H₂O (mg/mL); X₇: CaCl₂ (mg/mL); X₈: peptone (mg/mL); X₉: NH₄Cl (mg/mL) and X₁₀: urea (mg/mL).

Tab. 2. Screening of the investigated factors based on student *t*-test and *P*-value with the Plackett-Burman experimental design

Factors	Student <i>t</i> -test	<i>P</i> -value	Effect
PBM concentration (mg/mL)	3.99	0.0099	Positive
NH ₄ Cl (mg/mL)	-3.06	0.0227	Negative
Urea (mg/mL)	-2.17	0.0523	Negative
Inoculum age (h)	1.93	0.0735	Positive
KH ₂ PO ₄ (mg/mL)	1.60	0.1180	Positive
CaCl ₂ (mg/mL)	-0.67	0.4746	Negative
Inoculum size (% v/v)	0.67	0.5315	Positive
Peptone (mg/mL)	-0.52	0.6476	Negative
NaCl (mg/mL)	0.52	0.6487	Positive
MgSO ₄ ·7H ₂ O (mg/mL)	0.25	0.8241	Positive

3.3. Optimization of selected significant independent variables with a Box-Behnken design

In the present study a Box-Behnken experimental design was employed to determine the set of optimal values for the incubation temperature and initial pH of the culture broth together with the concentration of PBM and inoculum age that could maximize the amount of total extracted proteins by *A. keratiniphila* D2 from the keratinous waste substrate. The investigation was conducted according to the experimental layout presented in Table 3. By applying multiple regression analysis on the experimental data a relationship describing the independent and dependent variables and their mutual interaction was formulated. The experimental results were correlated with the help of a second order polynomial equation and the following quadratic model for the crude soluble protein extraction was derived:

$$Y = 13.24 + 0.35*A - 0.21*B + 6.13*C + 0.50*D - 0.016*AB + 0.88*AC + 0.092*BC + 0.67*AD - 0.51*BD - 0.11*CD - 0.77*AA - 0.29*BB - 1.93*CC - 0.50*DD$$

where Y is the concentration of crude soluble protein; A, B, C and D are in coded values of temperature, initial pH, PBM concentration and inoculum age respectively. The quadratic model describing the microbial degradation of thermally treated porcine bristles provided a very good approximation of the experimental data, as suggested by the high value of both the coefficient of determination ($R^2 = 0.981$) and the adjusted R^2 (0.958). In addition, in this model the terms AA, C, AC, CC and D were significant. Therefore, according to the obtained quadratic expression only three of the four independent variables, namely temperature (A), PBM concentration (C) and inoculum age (D), had a significant effect on the model response. Specifically, both the concentration of keratinous substrate (C) and inoculum age (D) had a linear and positive effect on the amount of total extracted proteins; nevertheless, C together with the temperature (A) showed a quadratic negative influence on the model response. Furthermore, a significant interaction between the terms A

and C was highlighted. In addition, when we consider the analysis of the standardized effects the following order of influence on the dependent variable could be inferred: $C > CC > AA > AC > D$.

Tab. 3. Box-Behnken experimental design for the investigated factors and observed responses

Run	A	B	C	D	Actual TEP* (mg/mL)	Predicted TEP* (mg/mL)
1	35	7.6	78.65	38	18.02 ± 0.54	17.90
2	30	7.6	44.59	38	13.43 ± 0.15	13.24
3	25	7.6	10.53	38	4.94 ± 0.12	4.93
4	30	8.4	44.59	48	12.11 ± 0.05	12.22
5	30	8.4	10.53	38	4.78 ± 0.04	4.58
6	35	7.6	10.53	38	4.79 ± 0.13	3.87
7	30	7.6	44.59	38	13.35 ± 0.32	13.24
8	35	7.6	44.59	48	13.43 ± 0.17	13.49
9	25	8.4	44.59	38	11.05 ± 0.25	11.63
10	35	7.6	44.59	28	9.52 ± 0.37	11.13
11	30	7.6	10.53	28	4.31 ± 0.08	4.06
12	25	7.6	78.65	38	14.65 ± 0.32	15.44
13	25	7.6	44.59	28	11.57 ± 0.17	11.78
14	25	7.6	44.59	48	12.79 ± 0.20	11.44
15	30	7.6	44.59	38	12.93 ± 0.84	13.24
16	30	8.4	44.59	28	12.57 ± 0.63	12.24
17	30	8.4	78.65	38	17.28 ± 0.97	17.03
18	25	6.8	44.59	38	12.25 ± 0.09	12.02
19	30	6.8	44.59	48	13.46 ± 0.83	13.67
20	30	6.8	10.53	38	4.67 ± 0.08	5.19
21	30	7.6	78.65	48	17.23 ± 0.45	17.34
22	30	7.6	78.65	28	17.54 ± 0.49	16.54
23	30	7.6	10.53	48	4.42 ± 0.02	5.28
24	30	6.8	78.65	38	16.80 ± 0.24	17.27
25	35	6.8	44.59	38	13.47 ± 0.58	12.75
26	35	8.4	44.59	38	12.20 ± 0.83	12.29
27	30	6.8	44.59	28	11.88 ± 0.51	11.64

*TEP = Total Extracted Proteins

Tab. 4. Analysis of variance (ANOVA) for the obtained regression model for the extracted soluble proteins

Source	Sum of squares (SS)	DF	Mean square (MS)	F-value	P-value
Model	483.56833	14	34.5406	43.2085	< 0.0001
A	1.45788	1	1.45788	1.8237	0.2018
B	0.54325	1	0.54325	0.6796	0.4258
C	451.56778	1	451.56778	564.8877	0.0001
D	3.04712	1	3.04712	3.8118	0.0764
AB	0.00100	1	0.00100	0.0013	0.9723
AC	3.08222	1	3.08222	3.8557	0.0732
BC	0.03416	1	0.03416	0.0427	0.8397
AD	1.81468	1	1.81468	2.2701	0.1578
BD	1.04449	1	1.04449	1.3066	0.2753
CD	0.04498	1	0.04498	0.0563	0.8165
AA	3.17899	1	3.17899	3.9768	0.0694
BB	0.44866	1	0.44866	0.5612	0.4682
CC	19.82055	1	19.82055	24.7945	0.0003
DD	1.34396	1	1.34396	1.6812	0.2191
Residual error	9.59273	12	0.7994	—	—
Lack of fit	9.4463834	10	0.944638	12.9099	0.0740
Pure error	0.1463431	2	0.073172	—	—
Core total	493.16105	26	—	—	—

$R^2 = 0.9805$ $R^2_{adj.} = 0.9579$

From the analysis of variance (ANOVA) it was possible to establish that the quadratic regression model was accurate as implied by the F score value of 43.2 (Table 4). In addition, there was only a 0.01% likelihood that such a “model F-value” could have occurred due to noise. The significance of the model was also confirmed by the results of the “lack of fit” test which were summarized in Table 4. After establishing that the quadratic model was capable to provide a relevant and accurate description of the design space which was in good accordance with the experimental data, the derived second-order polynomial equation was utilized to search for and select a set of optimal values for the cultivation parameters that could maximize the final concentration of total proteins extracted from the porcine bristle meal during the biodegradation process. Therefore, optimal values for the fermentation variables, namely 35 °C, a pH value equal to 6.9, 7% (w/w) PBM and an inoculum age of 48 hours, were predicted. As a consequence it was calculated that for these conditions the model forecast for the crude soluble protein concentration that could be extracted at the end of the microbial degradation process was 18.7 g/L. The surface plots illustrating the interactions among the four independent variables and their effect on the final concentration of total extracted proteins were provided in Figure 2. From the analysis of these graphs it was possible to confirm that the PBM concentration exerted the largest influence on the independent variable. In particular, it was clearly shown that the best choice for the keratinous substrate concentration coincided with the maximum level employed for this variable in the current experimental design. For instance, if we consider the plot describing the effect of the interaction of the PBM load with the incubation temperature on the amount of crude protein extracted it can be noticed how the PBM load dominates over temperature in reaching the convex optimum within the response surface obtained from these two independent variables.

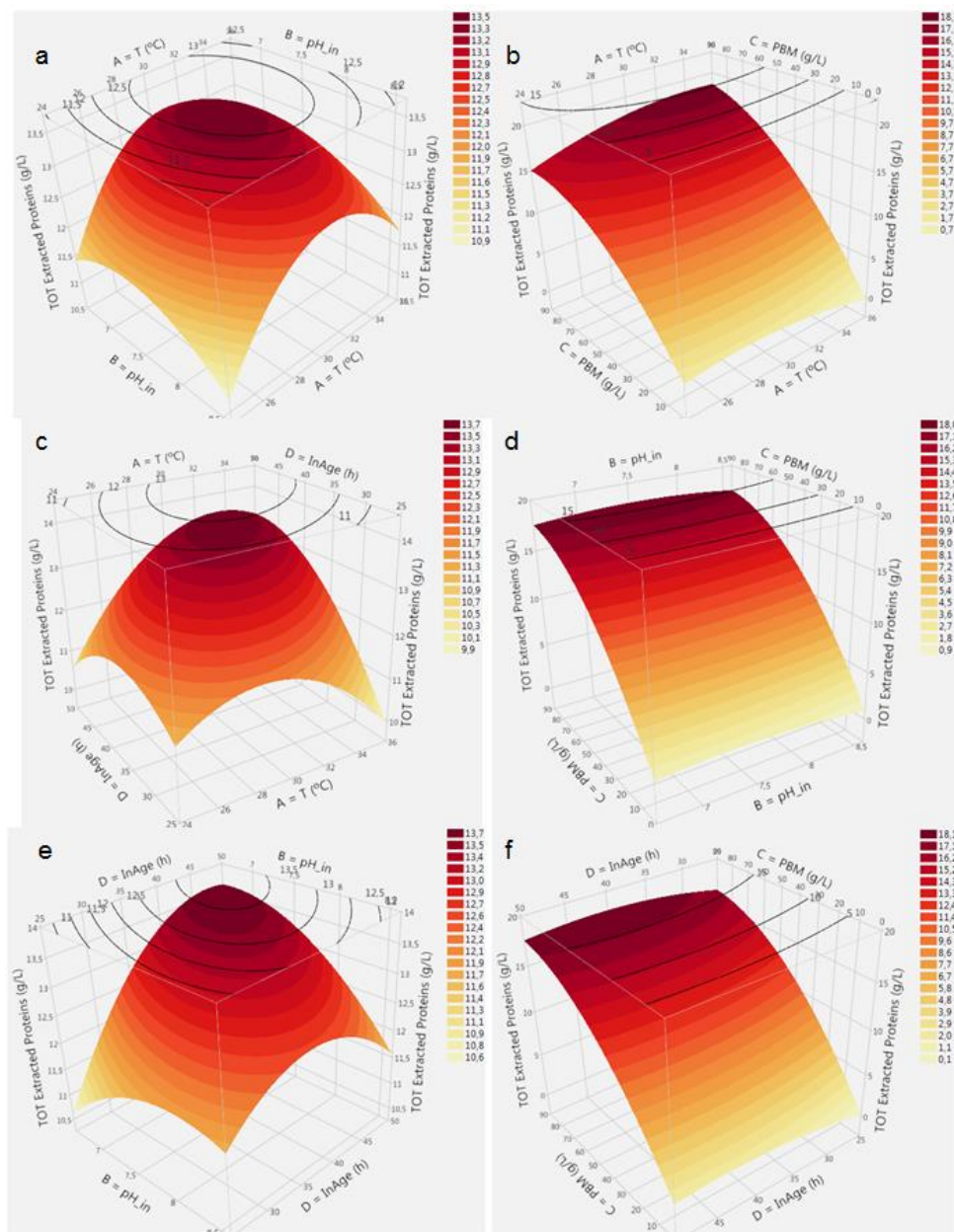


Fig. 2. Response surface plots for the concentration of totale extracted proteins (a-f) illustrating the interaction effects occurring among tested independent variables: (a) temperature vs initial pH, (b) temperature vs PBM concentration (c) temperature vs inoculum age (d) initial pH vs PBM concentration (e) initial pH vs inoculum age (f) inoculum age vs PBM concentration.

Moreover, in the same figure, it is also possible to appreciate the nonlinear influence, originating from the AC term of the quadratic model, that the fermentation temperature had on the amount of total extracted proteins. Interestingly, several reports have described the use of chicken feathers and sheep wool at concentrations up to 8% (w/v) in order to maximize the microbiological production of keratin protein hydrolysates (Embaby et al., 2010; Fakhfakh et al., 2011; Fakhfakh et al., 2013; Maciel et al., 2017). Nevertheless, it has been shown that the use of larger concentrations is not suitable for the effective degradation of raw feathers in a submerged fermentation system (Łaba et al., 2018). Indeed, in our case it was also observed that PBM loads larger than 7% (w/w) completely hampered the growth of *A. keratiniphila* D2 (data not shown). Al-Mussallam et al. (2003) indicated that the optimal cultivation temperature for *A. keratiniphila* D2 in a GYM medium was 28°C. Moreover, it has been shown that cell growth is one of the key factors affecting keratinases production (Riffel et al., 2011; Fang et al., 2013b). Besides, in Chapter 3 it was revealed that a rise in the catalytic rate of the keratinolytic pool of enzymes secreted by *A. keratiniphila* D2 was favoured by an increase of temperature, with the keratinolytic activity of the enzymatic cocktail reaching a maximum at 62°C. It follows that the effect of the temperature could be the result of the combination of two different and at the same time superimposed phenomena. At the beginning, in the interval in between 25 and 28°C, the rate of growth of the actinobacterium should increase, resulting in more microbial mass produced and, therefore, a larger amount of keratinolytic enzymes accumulated into the culture medium. Moreover, also the rate of keratinolysis should improve. Next, when we consider the interval above 28 °C and up to 35 °C, the growth rate of the filamentous organism should start to slow down, resulting in less microbial mass and, therefore, a lower amount of keratinases secreted into the culture broth. In addition, the rate of keratinolysis should still continue to increase within this interval. As a consequence, in the first part of the interval two positive contributions should be the driving force behind the detected increase in the extraction levels of proteins. On the

other hand, in the second part of the interval one negative contribution together with a positive one should be the reason for the plateau observed in Fig. 2b. The linear and positive effect that the variable D exerted on the model response suggested that the inoculum with the oldest age was the seed culture capable to maximize the final concentration of total proteins extracted during the biodegradation process. This was in contrast, for example, with the observations by Wang and Shih (1999) in the case of *B. licheniformis* PWD-1 for which a seed culture at early age was crucial to keratinase production during cultivation on 1% feather mineral medium. The microbial cultivations which were conducted during this optimization work, like the large majority of the experimental studies which have been described in the literature, were carried out in shake flask systems. The use of Erlenmeyer flasks for submerged cultivations implies the complete absence of pH control during the microbial degradation process. Therefore, the reported pH values, as in this case, correspond to the pH of the culture broth at the beginning of the fermentation process. Initial pH values are normally selected in accordance with the type of microorganism employed and values ranging from 6 to 10 have usually been reported (Sahoo et al., 2017). For instance, Kshetri et al. (2017) observed that the optimal initial pH for feather protein hydrolysate production by *Chryseobacterium sediminis* RCM-SSR-7 was 7.0-7.5, which was consistent with the value of 6.9 found in this study. On the contrary, Fakhfakh et al. (2011) reported that a very alkaline initial pH of 10 was the best choice in order to maximize the yield in feather protein hydrolysate obtained employing the keratinolytic bacterium *B. pumilus* A1.

3.4. Microbiological degradation of the keratin-rich waste material

The predicted optimal values for the fermentation variables were validated by means of a cultivation experiment conducted in duplicate. To this end, biodegradation of thermally pretreated porcine bristle powder for the production of keratin protein hydrolysate was carried out in a 3L laboratory scale stirred tank bioreactor inoculated with a seed culture of 48 hours.

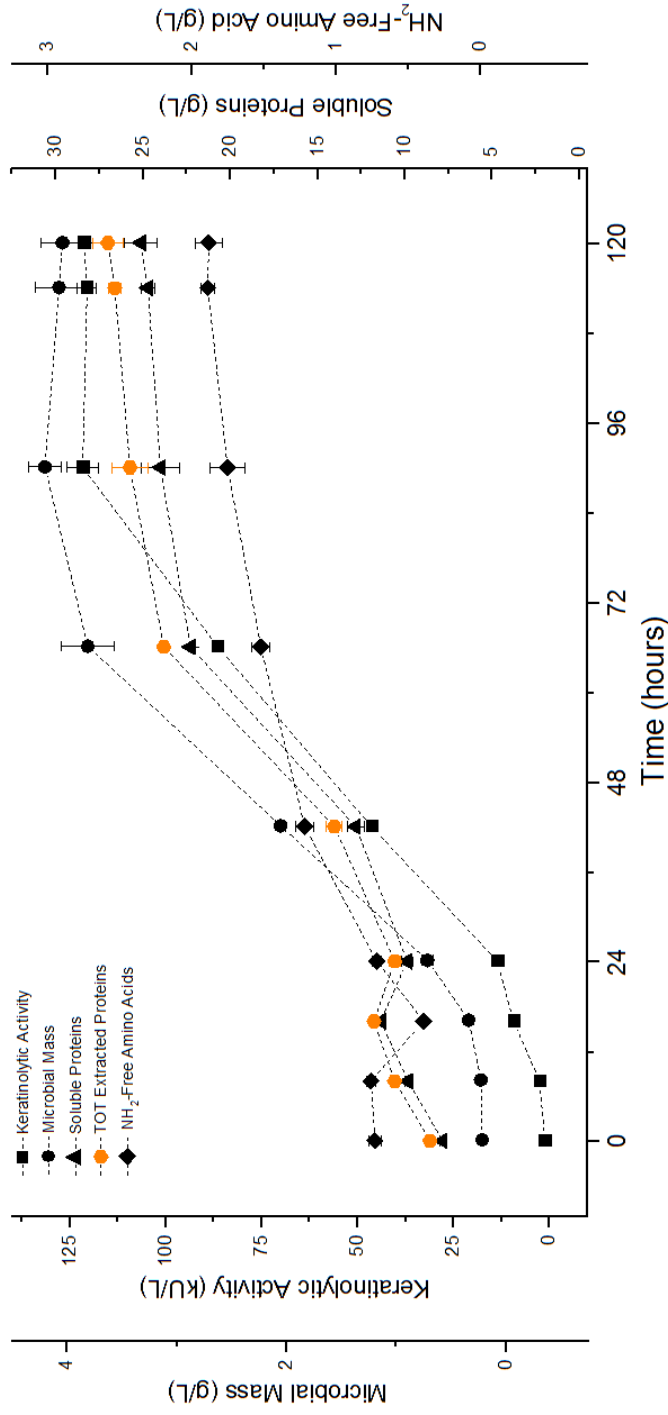


Fig. 3. Time course of PBM microbial degradation process by *Amycolatopsis keratiniphila* D2 in a 3-L bench-scale bioreactor as a function of cultivation time. Keratinolytic activity (■), microbial mass (●), crude soluble proteins (▲), NH₂-free amino acids (◆) and total extracted proteins (♦) TOT Extracted Proteins = Crude Soluble Proteins + NH₂-Free Amino Acids.

The cultivation temperature was regulated at 35 °C and 7% w/w pig bristle meal was utilized as the only source of C and N. In addition, the initial pH was adjusted to 6.9 and controlled around a setpoint value of 8.0 by acid addition. At the same time, the aeration rate was kept at 1.0 vvm to provide fully aerobic conditions ($DO \geq 30\%$), and meanwhile, the agitation rate was maintained constant at 500 rpm. As a result, during the batch cultivation of *A. keratiniphila* D2 in a 3L fermenter maximum keratinolytic activity on azokeratin was measured after 90 hours (122 ± 4.0 kU/L) of aerobic submerged fermentation (Figure 3). Furthermore, it was observed that the microorganism began to grow following a lag phase of approximately 8 hours before cells could start to propagate exponentially. The reason may lie in the fact that the inoculum was transferred from a complex GYM medium rich in all the nutrients needed by the microbial cells into a mineral basal medium where the only material from which C, N, and energy could be extracted by the cells was the keratinous particulate substrate. The microorganism ceased to grow exponentially after 42 hours of aerobic cultivation. Next, following a transitory phase of deceleration, the strain reached the early stage of the stationary phase after approximately 66 hours of fermentation run (3.8 ± 0.2 g/L). Through the course of the exponential phase, bacterial cells grew at a constant maximum specific rate of 0.066 h^{-1} . From 90 hours onwards the microbial strain fully entered its stationary phase where no net increase in the cell number was observed and a final microbial mass concentration of about 4.1 ± 0.2 g/L was attained. Maximal production of keratinolytic proteases was measured towards the end of the early stage of the stationary phase. In fact, the synthesis of microbial keratinases is generally regulated in accordance with the stage of development in which the cells are found, and it is often observed that a peak in enzyme concentration is reached at the end of the exponential growth and/or at the early stage of the stationary phase (Ramnani and Gupta, 2004; Joshi et al., 2008). Indeed, the described results clearly indicated that *A. keratiniphila* D2 synthesized keratinolytic enzymes as primary metabolites and that the production of its keratinases

was linked to the growth of the microorganism. Moreover, it was evident that the microbial cells could employ porcine bristle meal as the only source of C, N, and energy (Ramakrishna Reddy et al., 2017). As expected, keratinase synthesis was followed by the decomposition of the keratinous substrate and the subsequent release of soluble proteins, peptides, and amino acids into the culture supernatant. For instance, from Figure 3 it is possible to notice that, a steep increase in soluble protein concentration within the fermentation broth was detected in the first 24 hours from the start of the cultivation. Besides, after 66 hours of fermentation run, the rate of keratin hydrolysis decelerated significantly to eventually become very slow following about 90 hours from the start of the microbial degradation process. At the end of the fermentation, approximately 25.1 ± 0.9 g/L of crude soluble proteins were accumulated into the culture vessel. Even though a fraction of the solubilized keratinous substrate was converted into microbial biomass, in addition to the small percentage of crude soluble protein detected in the culture supernatant which were secreted by the microorganism as a consequence of its own metabolic activity, it is safe to state that the rapid increase in protein concentration observed after day 1 was primarily the result of porcine bristle microbial degradation. It is also worth mentioning that, keratinase production and keratin degradation do not necessarily take place at the same time. In fact, in our case keratin degradation occurred only after a certain amount of keratinolytic enzymes were already produced (Kothari et al., 2017). During the growth of *A. amycolatopsis* D2 on pig hair keratin a gradual increase in the pH of the culture medium was measured, starting from the initial value of 6.9. When a pH value of about 8.0 was reached, the peristaltic pump for the addition of the acidic buffer solution was automatically started, and the pH was stabilized around the final setpoint value of 8.0 which was then maintained constant up until the end of the fermentation process. It has been often described in the literature that a decrease in keratinase activity was observed after its peak value was reached. It has been usually speculated that the reason for this particular phenomena to occur might be the production,

concomitantly to the synthesis of keratin specific enzymes, of constitutive proteases that are normally active on a broader substrate spectrum and could target in an unselective fashion, in addition to other proteins, also the secreted keratinases causing their progressive inactivation (Lemes et al., 2016). Nevertheless, when considering the current study, following 90 hours of fermentation run no significant loss in keratinolytic activity was detected compared to the point at which the peak value was reached. It could be hypothesized that an excessive alkaline environment could be responsible for the consequent gradual inactivation of the particular set of keratinolytic proteases secreted by *A. keratiniphila* D2. Therefore, the stabilization of the pH around a value of 8.0 could have had a positive effect on the retention of the catalytic activity of keratinases especially after the secretion of the keratinolytic enzymes had been halted by the microbial organism. The highest levels of free amino acids released inside the culture broth were recorded towards the end of the microbial biodegradation process. Indeed, after 120 hours from the start of the fermentation run a concentration of approximately 1.9 ± 0.1 g/L of NH_2 -free amino acids was measured within the culture supernatant. From a biocatalytic point of view, the release of amino acids is normally brought about by the action of oligopeptidases that cleave the oligopeptides obtained after the action exerted by the exopeptidases on the soluble protein extracted during the microbial degradation process. Indeed, the microbiological breakdown of keratinous substrates is the result of a complex, and still not well understood, set of events involving the participation of several different keratin-specific proteases acting synergistically rather than the work of a single keratinolytic enzyme (Huang et al., 2015; Lange et al., 2016; Sharma and Devi, 2018). Therefore, the sum of the crude soluble proteins and NH_2 -free amino acids which were recovered within the culture supernatant could be considered as a measure of the total proteins extracted from the keratinous solid biowaste throughout the course of the biodegradation process. The value should not include the crude soluble proteins and NH_2 -free amino acids which were already present into the

culture broth at the start of the fermentation run since they were released from the solid protein-rich material during the autoclavation of the culture medium. Consequently, a final 18.5 g/L of bristle protein hydrolysate was extracted from the keratin-rich substrate during the course of the microbial decomposition of the PBM in the bench-scale stirred tank fermenter. Thus, the concentration of total extracted proteins that was measured at the end of the experimental test was nearly equivalent (i.e. 98.9% of the predicted result) to the previously estimated value (18.7 g/L) which was obtained with the help of the quadratic model derived during the course of the statistical optimization of the most significant cultivation variables.

4. Conclusion

In the present study, microbial degradation of porcine bristle meal by the keratinolytic filamentous bacterium *A. keratiniphila* D2 for the production of bristle protein hydrolysates was successfully optimized by means of statistical methodologies such as Plackett-Burman and Box-Behnken experimental designs. In the first place, the optimal levels of the four most significant fermentation variables, i.e. a cultivation temperature of 35 °C, pH value equal to 6.9, 7% (w/w) PBM and an inoculum age of 48 hours, were predicted by means of a regression model. Then, the obtained set of optimized cultivation parameters was validated by means of a duplicate experiment conducted in an aerated 3-L bench scale stirred tank bioreactor. The concentration of total extracted proteins (18.5 g/L) measured in the obtained final bristle protein hydrolysate was in very good agreement with the value which was predicted by the quadratic model (18.7 g/L). Finally, it could be suggested that keratin protein hydrolysates obtained through microbial conversion of recalcitrant hair-type keratinous by-products, such as the porcine bristle meal utilized in this study, have the potential to be employed as a protein supplement for animal feed formulations.

Acknowledgments

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Supporting Figures:

S1:

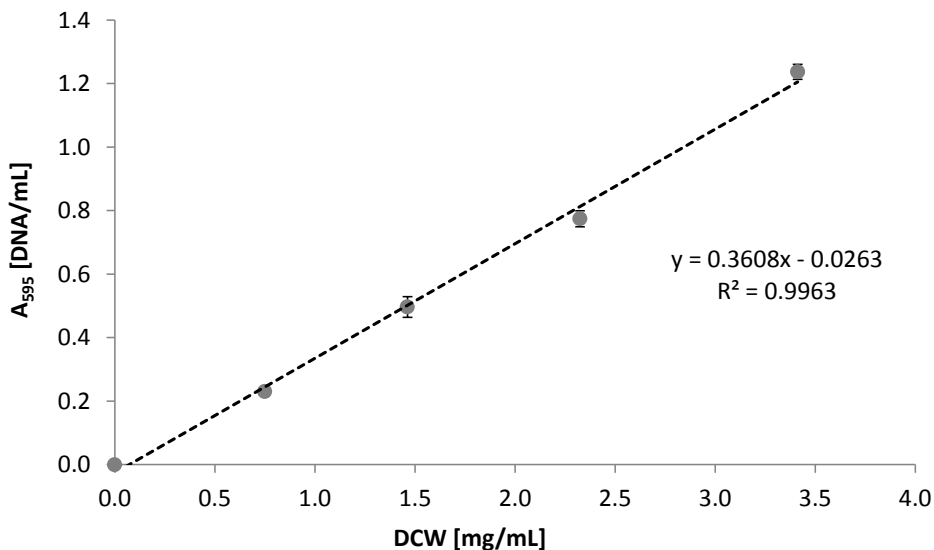


Figure S1: Calibration curve correlating *Amycolatopsis keratiniphila* D2 dry cellular weight (DCW) to the absorbance measured at 595 nm and obtained employing the simplified diphenylamine DNA quantification method. In order to obtain a reliable and accurate quantification of microbial cell growth in a cultivation medium containing solid keratin particles it was chosen to employ the DNA extraction and quantification method developed by Zhao et al. (2013). The diphenylamine colorimetric method to quantify microbial DNA showed a very good correlation with the measurement of cell density obtained with a standard cell dry weight method.

Chapter 3

An integrated strategy for the cost-effective production of bristle protein hydrolysate by the keratinolytic filamentous bacterium *Amycolatopsis keratiniphila* D2.

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Abstract

In a conventional keratin microbial degradation process keratin-specific proteases production and hydrolysis of keratinous residual biomass are both achieved during the same stage of the process which in turn runs under suboptimal conditions. In this study the keratinolytic actinomycete *Amycolatopsis keratiniphila* D2 was successfully employed to biodegrade pretreated pig bristles at high solid loading (16 % w/v) with an overall accumulation of 89.3 g/L protein-rich hydrolysate and a productivity of 427 mg of crude soluble proteins per litre per hour. The two-stage submerged fermentation process developed in this work enabled to recover, in a single unit operation, approximately 72.5 % of the protein material contained in the keratin-rich biowaste structure. The obtained protein hydrolysate powder displayed a 2.2 fold increase in its *in vitro* digestibility with respect to the non-hydrolysed pretreated substrate. The soluble proteins, peptides and free amino acids recovered during the enzymatic extraction process represent a viable alternative protein source in animal feed.

Abbreviations

PBM porcine bristle meal

BPH bristle protein hydrolysate

SEC size exclusion chromatography

1. Introduction

Keratins are fibrous structural proteins containing a high number of disulfide bonds in their primary structure (Wang et al., 2016), which makes them water-insoluble and resistant to lysis with common proteolytic enzymes (Brandelli et al., 2010). Keratin-rich animal by-products such as chicken feathers, horns, and bristles are the third most abundant renewable polymeric material present in nature after cellulose and chitin (Lange et al., 2016). Keratin waste biomass is classified as a low-risk animal byproduct which occurs abundantly in slaughterhouses and meat & poultry processing plants. Therefore, this typology of solid residue is not suitable for human consumption and needs to be treated before its disposal into the environment (Korniłowicz-Kowalska and Bohacz, 2011). Based on their secondary structure, keratins are divided into α - and β -keratins. The α -type is also named hard keratin, and that is because it contains a higher cysteine content which allows the formation of a larger number of disulfide bridges between cross-linking protein chains (Wang et al., 2016). Some bacteria, actinomycetes and keratinophilic fungi have the capability of using keratin as the only source of C, N, S and energy. Specifically, they are able to synthesize microbial keratinases (EC 3.4.21/24/99.11), which are enzymes belonging to the class of serine proteases or metalloproteases, showing high levels of hydrolytic activity towards keratinous substrates. Therefore, biodegradation of keratin waste with keratinolytic bacteria can be considered an attractive way of transforming it into products of practical industrial value (Kothari et al., 2017; Korniłowicz-Kowalska and Bohacz, 2011, Daroit and Brandelli, 2014). Protein hydrolysates obtained from the biodegradation of keratin-rich animal processing by-products have a potential for replacing a considerable fraction of the fish meal used in aquaculture feed formulation and more in general, for becoming a protein supplement in animal feed (Martínez-Alvarez et al., 2015; Mazotto et al., 2017). For instance, only in

2013, approximately 222,000 tons of wet pig bristles were generated as a waste inside the European Union (Gachango et al., 2017). The effective reutilization of such a type of keratinous by-product constitutes an unsolved problem due to its outstanding resistance to chemical and enzymatic digestion in comparison to softer categories of keratins such as those present inside the structure of chicken feathers (Łaba et al., 2015a; Brandelli et al., 2015). Therefore, in the present work, we focus on the recovery of valuable proteins from thermally pretreated pig bristles using the keratin-degrading *Amycolatopsis keratiniphila* D2 strain previously isolated by Al-Musallam et al., (2003). Specifically, we demonstrate and optimize a novel two-stage process, for the efficient production of keratinolytic enzymes and the effective hydrolysis of pretreated pig bristles at high dry solids loadings (16 % v/w) in a standard stirred tank aerobic bioreactor.

2. Material and Methods

2.1. Porcine bristle meal

The slaughterhouse by-product used as C, N and energy source for microbial growth and employed as well to prepare azokeratin for activity assays was kindly supplied by DAKA SARIA Group A/S (Løsning, Denmark). The keratinous residual material consisted mainly of porcine bristles, which were chopped, thermally pretreated (150 °C, 6 bars, 20 min), dried and finally crushed into smaller particles.

2.2. Chemicals and gases

All chemicals employed in this study were of analytical grade and were purchased from Sigma Aldrich ApS (Brøndby, Denmark); gases were supplied by AGA A/S (Copenhagen, Denmark).

2.3. Microorganism and media composition

The strain *Amycolatopsis keratiniphila* D2 (DSM 44409) was obtained from DSMZ (Braunschweig, Germany). Seed culture glycerol stocks were prepared and stored at -80 °C prior to use. *A.*

keratiniphila D2 was cultivated in mineral keratin (Basal) medium formulated according to the following composition (g/L): 0.75 NaCl, 1.75 K₂HPO₄, 0.25 MgSO₄·7H₂O, 0.055 CaCl₂, 0.010 FeSO₄·7H₂O, 0.005 ZnSO₄·7H₂O, 10.53 (1 % w/w) PBM powder. The medium was sterilized at 121 °C for 20 min before use.

2.4. Inocula preparation

Inocula were prepared by propagating frozen microbial cell stocks of *A. keratiniphila* D2 in Erlenmeyer flasks (500 mL) containing 100 mL of GYM medium (4.0 g/L glucose, 4.0 g/L yeast extract, 10.0 g/L malt extract); the initial pH of the culture broth was adjusted to 7.2 before autoclaving. Each flask was inoculated with 1 mL of a glycerol stock (20 % v/v) of bacterial cells and incubated on an orbital shaker (200 rpm) at 28 °C for 48 hours. Microbial cells were harvested by centrifugation (4000 rpm for 20 min at 4°C), washed twice in sterile phosphate buffer saline (PBS) solution (15 mM NaCl, pH 7.2) and again resuspended in an appropriate volume of PBS solution.

2.5. Aerobic shake flask cultivations

Aerobic shake flask experiments were conducted in 250 mL Erlenmeyer flasks each one containing 50 mL of mineral keratin medium. After the initial pH of the culture broth was adjusted around a value of 7.2, 1 mL of resuspended bacterial cells was inoculated to obtain a microbial concentration of about 0.15 mg_{cdw}/mL at the start of the cultivation process. All shake flask experiments were conducted in triplicate.

2.6. Keratinase assay

Crude keratinase activity was assayed on supernatant obtained after centrifugation (12000 × g, 5 min) of the fermentation broth with azokeratin as a substrate by the following method. The reaction mixture contained 200 µL of enzyme preparation and 1400 µL of 10 g azokeratin l-1 in 50 mM tris buffer, pH 8.0. The mixture was incubated for 15 min at 50 °C; the reaction was stopped by the addition of 1600 µL Trichloroacetic acid (TCA) to a final concentration of 100 g/l. After centrifuga-

tion at $10,000 \times g$ for 5 min, the absorbance of supernatant was determined at 440 nm. One unit of enzyme activity was the amount of enzyme that caused a change of absorbance of 0.01 at 440 nm after 15 min incubation at 50 °C.

2.7. Crude soluble proteins determination

Soluble protein concentration was determined by the bicinchoninic acid (BCA) assay (Smith et al., 1985) employing bovine serum albumin (BSA) as protein standard (Pierce™ BCA Protein Assay Kit, Thermo Scientific).

2.8. Optimization of process parameters

Cultivation parameters were optimized by a one-variable-at-a-time (OVAT) approach. *A. keratiniphila* D2 displayed efficient keratin degradation in a medium containing PBM powder as only source of carbon and nitrogen. In particular, both incubation temperature (25, 28, 30 and 35 °C) and PBM powder concentration (1, 2, 3, 4, 5, 6, 7 % w/v) were optimized considering both the level of keratinolytic proteases secreted and the amount of crude soluble proteins extracted. The optimal reaction temperature of the cell free keratinase extract from *A. keratiniphila* D2 was investigated in the temperature range 28–88 °C in 50 mM TRIS buffer at pH 8.0, whereas the optimal pH tests were conducted at values ranging from 5 to 11, employing 1 % v/v azokeratin powder as substrate.

2.9. Laboratory scale batch cultivation

Batch submerged cultivation of filamentous bacteria *A. keratiniphila* D2 were carried out in duplicate in two identical 3-L bioreactors (Applikon, Delft, The Netherlands), with a 2-L working volume. Both fermenters were equipped with two 6-bladed Rushton impellers. In the first stage of the fermentation process, cultivation parameters were optimized in order to maximize keratinolytic enzyme secretion: the initial pH of the keratin basal medium was adjusted to 7.2; the temperature was set at 28 °C; fully aerobic conditions (dissolved oxygen concentration ≥ 30 %) were ensured by flowing 1 vvm of air throughout the vessel; stirring was kept at a constant rate of 500 rpm. Fer-

menters were inoculated with 2.5 % (v/v) of fresh inoculum ($\approx 0.35 \text{ mg}_{\text{cdw}}/\text{ml}$) and run under the previously described condition for up to 94 h. During the course of the cultivation of *A. keratiniphila* D2 in a keratinous waste containing medium an increase of pH was observed. Once a value of 8.0 was reached, in order to avoid a further increase of alkalinity in the culture broth, the pH was stabilized around a set point value of 8.0 and then controlled by adding 2 M H_2SO_4 with a peristaltic pump. In the second stage of the bioprocess, cultivation parameters were optimized in order to maximize both the rate of keratin degradation and the extent of crude protein extraction and solubilisation as well: the temperature was raised and maintained at 50 °C; no more air was supplied to the reactor (dissolved oxygen concentration $\approx 0 \%$); the stirring rate was increased to 800 rpm; the automatic addition of a 6 M Na_2OH solution prevented acidification of the hydrolysate and kept its pH around a set point value of 8.0. Microbial mass concentrations were determined according to the method developed by Zhao et al. (2013) (see Supporting Figure S1). The second stage of the process was carried out for about 112 h. At the end of the two stage process, the protein hydrolysate was separated from the residual non-solubilized keratin and spent microbial mass by centrifugation at 4000 rpm for 40 min (Heraeus™ Multifuge™ X3R, Thermo Fisher Scientific). In addition vacuum filtration was employed to further remove small insoluble particles from the protein digest down to an average particle size of 12-15 μm (Sartorius™ Grade 288 Qualitative Filter Papers Disc). Finally the bristle protein hydrolysate was lyophilised in a CoolSafe™ 4 L freeze dryer (LaboGene, Denmark) in order to obtain a dry powder.

2.10. Amino acid analysis and in vitro digestibility of bristle protein hydrolysate

Freeze dried samples were shipped to Eurofins Agrosience Services A/S, Middelfart, Denmark, <https://www.eurofins.com/agrosienceservices> for total nitrogen, amino acid analysis and for performing an *in vitro* pepsin digestibility test. Samples were both analyzed for total amounts of amino acid (acid hydrolysis of samples) and free amino acid content (no acid hydrolysis of samples).

2.11. Size exclusion chromatography of hydrolysate

Hydrolysate from the end of the hydrolysis process (206 h) was used for size analysis. The sample was prepared by filtration through a 0.22 μm filter (Minisart NML, Sartorius™), followed by centrifugation (15 min, 10,000 g). The supernatant was diluted ten times into the running buffer (10 mM $\text{NH}_4^+(\text{CH}_3\text{COO}^-)$, 30% acetonitrile in water at pH 9 and then centrifuged again (15 min, 10,000 g). A Superose® 12 10/300 column was equilibrated with running buffer (10 mM $\text{NH}_4^+(\text{CH}_3\text{COO}^-)$, 30% acetonitrile in water at pH 9 and then 1 ml of the diluted sample was loaded inside the column. The flow rate was set to 1 ml/min, separation was monitored by measurement of absorbance at 280 nm and the size separated sample was collected in 0.5 mL fractions. Calibration of the Superose® 12 10/300 column was performed by two consecutive runs of a mixture of calibration samples. The first run consisted of Aldolase (36 kDa), Chymotrypsinogen (25.6 kDa) and vitamin B¹² (1.3 kDa). The second run consisted of Thyroglobulin (660 kDa), Ovalbumin (66 kDa) and Ribonuclease (13.7 kDa). Furthermore, a measurable change in conductivity was considered as an indicator for elution of the smallest possible molecules. The calibration was performed in a different buffer than the one used for the sample (10 mM NaPi, 500 mM NaCl at pH 7.5). The molecular mass of the calibrants (kDa) was Log_{10} transformed and plotted as a function of elution volume (K_e), after that a linear model was fitted to the data ($\text{Log}_{10}(\text{mass}) = a * K_e + b$). The column was further calibrated by running free amino acids (Gly, Leu, Arg, Phe and Trp) over the column to test whether these would adhere to the column material when using the running buffer used for the sample runs.

2.12. Size estimation of fractionated hydrolysate using gel electrophoresis

Fractions from the sample run were used for size determination using the Nu-PAGE gel electrophoresis system (Invitrogen™, Novex™, Thermo Fisher Scientific). Half of the volume from the fractions (250 μl) was dried using speed vacuum drying, after which the dried material was dissolved in sample buffer mixed with reducing agent and heat treated (90 °C, 5 min). Samples were run on a

Nu-PAGE bis-tris 4–12 % gel and stained using a coomassie blue based stain. The protein ladder used was Mark 12™ (Thermo Fisher Scientific).

2.13. Statistical analysis

Experimental data were analysed for statistical significance ($p < 0.05$) using one-way ANOVA, followed by Tukey's honestly significantly different (HSD) test.

3. Results and discussion

3.1. Effect of cultivation temperature and PBM concentration on keratinase secretion and crude protein extraction during PBM biodegradation

Optimal cultivation conditions for enhanced keratinase secretion levels can differ significantly according to the type of microorganism under investigation. It is therefore necessary to conduct a set of experiments in order to establish those conditions which maximize keratinolytic enzyme production for the strain studied here. The actual capacity of the employed keratin-degrading strain to accumulate large quantities of extracellular keratinases in the culture medium has a direct effect on the rate of hydrolysis of the pretreated keratinous waste and, consequently, on the effective enzymatic extraction of digestible proteins (Fakhfakh et al., 2011; Fakhfakh et al., 2013; Fontoura et al., 2014). Given these facts, process parameters were investigated in order to identify the set of cultivation conditions which could maximise the process metrics (i.e., titre, extraction yield and volumetric productivity) in terms of production rate of soluble proteins.

It has been widely described that the synthesis of extracellular microbial keratinases can be induced by the presence of keratinous material in a basal mineral medium (Brandelli et al., 2010). Additionally, it has been also documented that the secretory levels of keratinolytic proteases could be improved by supplementing the culture medium with non-keratinous substrates (Fang et al., 2013b; Daroit et al., 2011). Nonetheless, one of the major obstacles preventing large scale production of microbial keratinases at an industrial level is the possibility to obtain these biocatalysts in sufficient-

ly large amounts and at a competitive manufacturing cost. Therefore, in this study we only employed inexpensive keratin-rich waste material, namely pig bristles meal, to achieve the cost effective synthesis of microbial keratinase.

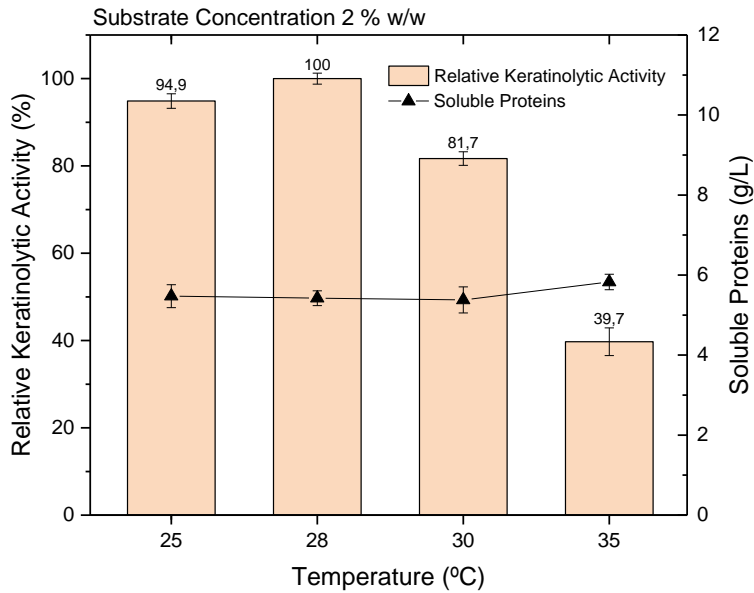


Fig.1. Effect of temperature on keratinase secretion and crude protein extraction during 2 % (w/w) PBM degradation by *A. keratiniphila* D2.

Several actinobacteria capable of producing keratin degrading enzymes have been reported and described in the literature (Meng et al., 2007; Gong et al., 2015; Ningthoujam et al., 2016; Habbeche et al., 2014). The capability of *A. keratiniphila* D2 to decompose keratin was tested by growing the strain on mineral medium containing 2 % (w/w) PBM. The effect of temperature on the quantity of keratin-specific proteases secreted by *A. keratiniphila* D2 was studied in the first place. The highest amount of keratinases accumulated in the culture broth was invariably reached after 4 days of cultivation. Maximum keratinolytic enzyme secretion was observed at 28 °C (120 ± 15 U/mL) and

significantly decreased above this temperature (Fig. 1). This finding can be ascribed to the fact that the strain grew better at this temperature having the highest maximum specific growth rate (data not shown). In general some extracellular proteolytic enzymes are constitutively expressed by the microorganism. This means that the amount of microbial mass obtained at the end of the exponential growth phase could regulate the final level of protease accumulated in the culture broth. It is therefore possible that conditions which maximize growth rate will yield larger quantities of keratinolytic enzyme at the end (Daroit and Brandelli, 2013; Verma et al., 2017).

A plateau in the amount of crude protein extraction was observed after 4 days of cultivation for each of the four tested temperatures (Fig. 1). Despite the decrease in keratinolytic enzyme secretion with the increase of temperature a stable production of crude soluble protein was observed with even a small increase in protein hydrolyseate productivity for the case of 35 °C (5.83 ± 0.19 g/L). This was explained by the fact that the catalytic rate of the enzymatic pool of keratinases increased with the temperature reaching a maximum at 64 °C (Fig. 3). The activity of the enzymes was gradually increased from 25 to 35 °C resulting in a stable rate of hydrolysis despite the decrease after 28 °C in the amount of secreted keratinases.

The concentration of pretreated bristles powder contained in the culture media directly affected the level of keratinolytic proteases secreted by *A. keratiniphila* D2 during the course of its aerobic growth in a shake flask at 28 °C (Fig. 2). Maximum keratinase activity was always observed after 4 days of cultivation. In addition, PBM concentration affected only the maximum amount of keratinolytic enzyme secreted by the cells, but did not have a significant effect on the time needed to reach its concentration peak. The amount of keratin-specific proteases produced by *A. keratiniphila* D2 was inversely correlated to the concentration of keratinous substrate when the latter was increased from 1 to 3 % (w/w). This finding is consistent with what was previously reported by Reddy et al. (2017) in the case of *Bacillus pumilus* GRK, or similarly Cheng et al. (1995) regarding *Bacillus*

licheniformis PWD-1. In addition, no substantial decrease in keratinases production was observed after the concentration of PBM was raised further from 4 to up to 7 % (w/w). A plateau in the production of crude soluble proteins was reached after 4 days for 1, 2, 3, 4 % (w/w) PBM concentration; 5 days in the case of 5 % (w/w) PBM; 6 days for 6 % (w/w) and 7 days for 7 % (w/w). The crude soluble protein titer increased with increasing bristle meal concentration reaching a maximum at 7 % (w/w) PBM ($17.9 \text{ g}_{\text{CSP}} \cdot \text{L}^{-1}$). The highest productivity in terms of crude soluble proteins was achieved with 4 % (w/w) PBM ($117.3 \pm 0.7 \text{ mg}_{\text{CSP}} \cdot \text{L}^{-1} \text{ h}^{-1}$), while the maximum yield was reached with 1 % (w/w) PBM ($39.1 \text{ g}_{\text{CSP}}/\text{g}_{\text{PBMdw}}$).

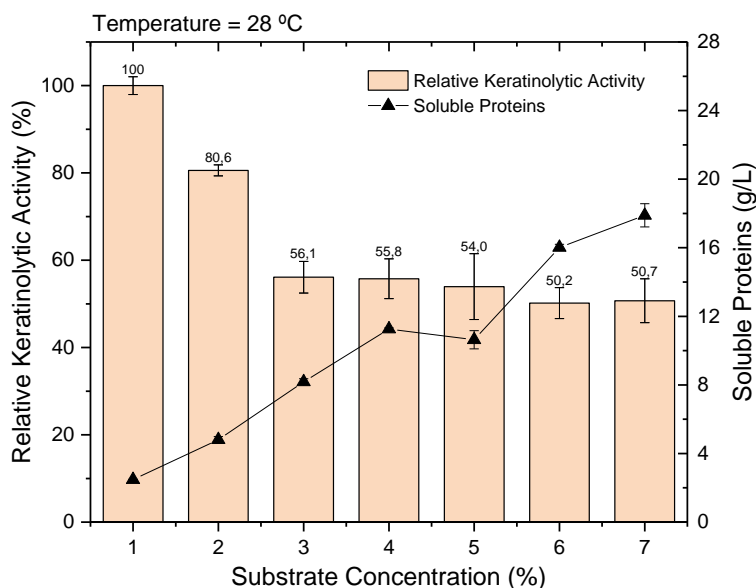


Fig.2. Effect of PBM concentration on keratinase secretion and crude protein extraction during PBM degradation by *A. keratiniphila* D2 at 28 °C.

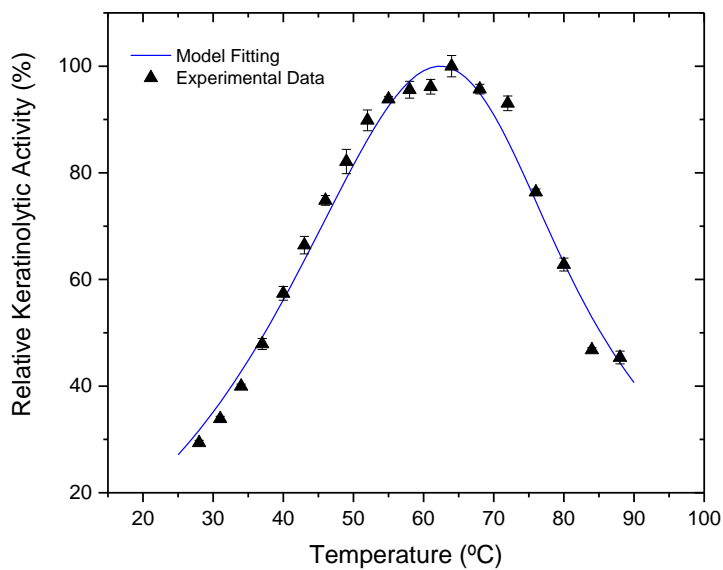


Fig. 3. Effect of temperature on enzymatic activity of the keratinolytic proteases cocktail produced by *A. keratiniphila* D2.

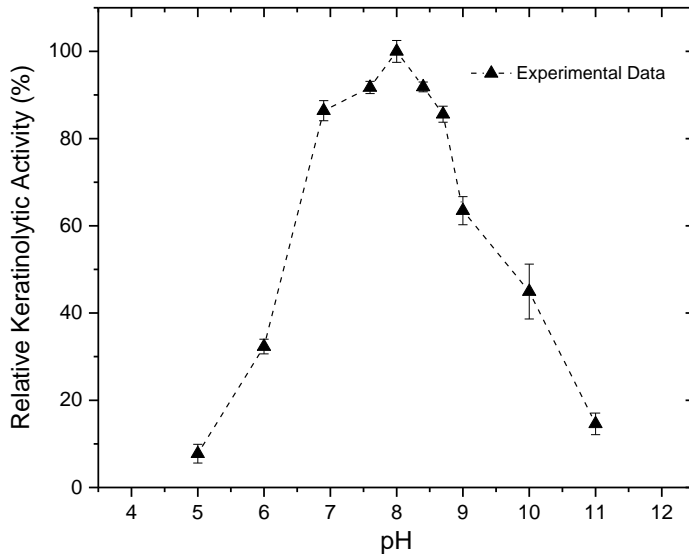


Fig. 4. Effect of pH on enzymatic activity of the keratinolytic proteases cocktail produced by *A. keratiniphila* D2.

A. keratiniphila D2 is a mesophilic strain; nevertheless it was observed that its keratinases have a global optimum in the thermophilic temperature range. Moreover, like most of the keratin-degrading actinomycetes, *A. keratiniphila* D2 showed an optimum pH in the neutral/alkaline range (Daroit and Brandelli, 2014). Indeed, the highest keratinolytic activity was observed at pH 8.0 and about 62 °C (Fig. 3, Fig. 4). The activity of the cell-free crude keratinases extract decreased rapidly at temperatures lower than 46 °C and higher than 76 °C, with a reduction to 75 and 76 % respectively. In addition, regarding the effect of pH on the keratinolytic activity of the enzymatic cocktail a drastic decrease of its catalytic activity was observed outside the pH range 6.9 – 8.7; more specifically, at pH 6 and 9 a reduction of 32 and 64 % was detected, respectively.

3.2. Two-stage PBM biological degradation process

After the initial studies were carried out at the shake flask level, it was decided to transfer the biodegradation process to a bench-scale fermenter in order to investigate, in a more controlled environment, the effect of cultivation parameters such as agitation, aeration rate, dissolved oxygen concentration, pH and temperature, both on the secretion levels of keratin-specific proteases and on the amount of crude soluble proteins which could be extracted from the keratinous waste material and accumulated into the bioreactor. With this in mind, biodegradation of pretreated pig bristles for the production of keratin protein hydrolysate by *A. keratiniphila* D2 was carried out in a 3-L laboratory scale aerobic fermenter. In order to simultaneously maximize extraction yield and volumetric productivity an integrated strategy, consisting of a two-stage biodegradation process, was developed (Burg et al., 2016; Klamt et al., 2017). From a phenomenological point of view the aim of this approach was to completely decouple and, therefore, disengage the keratinolytic enzyme production phase from the keratin hydrolysis stage. The first stage of the process consisted of the production of the crude microbial keratinases by simply culturing *A. keratiniphila* D2 under conditions that maximize cell growth. Specifically, keratinolytic enzymes will be mainly synthesized and secreted during the late exponential and stationary phase of the growth of the microorganism. Therefore, throughout the initial stage, no attempt was made to try to balance the rate of formation of extracellular keratin-specific proteases with the rate of keratin enzymatic hydrolysis by compromising in between biomass growth and protein hydrolysate production. The soluble proteins extracted from porcine bristles were only considered as a nutrient source (i.e., the substrate) capable to meet the nutritional requirement of the microorganism during its growth. Part of the solubilized proteins, after further breakdown into peptides and amino acids, were consumed in order to provide the necessary nutrients required for the growth of the microorganism.

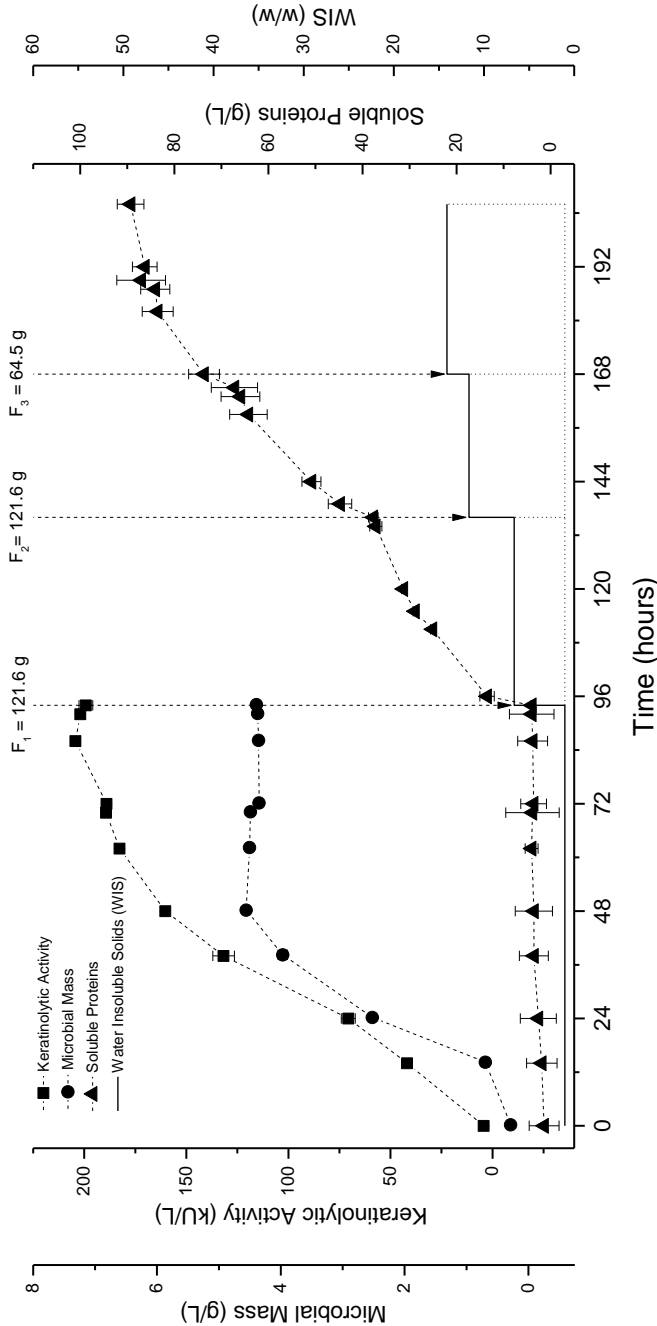


Fig. 5. Time course of two-stage PBM biological degradation process in 3-L bench-scale aerobic fermenter. Keratinolytic activity (■), microbial mass (●), soluble proteins (▲), and water insoluble solids (—).

Consequently, in order to attain optimal growth under fully aerobic conditions and therefore maximum rate of keratin-specific proteases production during the first phase of the process, the cultivation temperature was set at 28 °C and 1% w/w bristle meal powder was employed as the sole source of C and N. Moreover, the initial pH was adjusted to 7.2 and controlled around a set point value of 8.0 by acid addition. At the same time, the aeration rate was maintained at 1.0 vvm to provide fully aerobic conditions ($DO \geq 30\%$), and meanwhile the agitation rate was kept constant at 500 rpm. As a result, during the batch fermentation of *A. keratiniphila* D2 in a 3-L fermenter maximum keratinase production was achieved after 86 hours of aerobic submerged cultivation (204 ± 2.3 kU/L) (Fig. 5). This value was considerably higher than the one obtained in a shake flask run under the same conditions (i.e., 148 ± 3.0 kU/L). The reason for this observed diminished keratinase synthesis and/or secretion in a shake flask system with respect to the stirred tank bench-scale aerobic fermenter might be the result of poor agitation, due to insufficient mixing both of the keratin-medium constituents and microbial cells and, consequently, also a lack of uniformity in oxygen distribution in the reactor. (Zaghloul et al., 2011b).

The end of the microbial growth was observed after 48 hours; specifically, before entering the stationary phase, a final microbial mass concentration of 4.6 g/L was reached. *A. keratiniphila* D2 secreted extracellular keratinases before the start of the exponential growth phase and exhibited considerable keratinase secretion also after the end of the exponential growth. In particular, the specific rate of keratinase production was higher during the pre-exponential and stationary phases and lower during the exponential phase of growth of the microorganism (Daroit et al., 2011; Stiborova et al., 2016). After 62 hrs no significant increase in soluble protein extraction was observed (4.1 ± 0.1 g/L).

The cocktail of crude keratinolytic enzymes produced by *A. keratiniphila* D2 during the first phase was then employed in the second stage for the hydrolysis of the pretreated pig bristles. As a

consequence, in the second phase keratin waste material was not considered anymore as an inducer and solubilized proteins were no more seen as a substrate for microbial growth. The bioprocess conditions at this stage were optimized with the objective of drastically enhancing the rate of hydrolysis of the recalcitrant keratinous waste. Therefore, in the second phase, the temperature was increased to a constant value of 50 °C while pH was kept stable at 8.0. It is important to notice that, by doing so, the catalytic rate of the keratinolytic enzymatic pool was increased from about 29 % of its maximum value to approximately 85 % of the maximum value (Fig. 3). Additionally, no more air was supplied to the cultivation vessel, while the agitation rate was raised to 800 rpm. The actinobacterium was an obligate aerobic microorganism which was not capable of showing any further growth both in the case of a cultivation temperature of 50 °C and in the absence of oxygen. Therefore, the further consumption of precious nutrients, especially free amino acids and small peptides, was prevented during the second phase.

Regarding the economic feasibility of the microbial degradation process it is preferable to perform the enzymatic hydrolysis of keratinous waste biomass at high solids loadings. This permits to obtain keratin hydrolysates with higher titres of crude soluble proteins while reducing the capital and operating costs of the biological extraction step. Nevertheless, to run the second stage of the process in a batch mode is not convenient from an operational point of view. In fact, high initial levels of insoluble solids do not necessarily mean a linear increase in the crude protein extraction yield (Modenbach and Nokes, 2013; Kristensen et al., 2009). For the aforementioned reasons, aiming at increasing the final titer of crude soluble proteins accumulated during the keratin waste hydrolysis process, the cultivation was followed by a three step addition of extra keratin substrate (i.e.: 121.6 g dw at 94 h, 121.6 g dw at 136 h and 64.5 g dw at 165 h). The cumulative concentration of keratinous substrate added during the enzymatic hydrolysis of the bristle meal was 164.3 g/L dw. In analogy with a fed-batch cellulose hydrolysis process, the advantage of employing such a stepwise

addition of bristle meal powder was to give the possibility to keep the concentration of non-hydrolyzed particulate keratinous material at a manageable level during the course of the enzymatic extraction process (Hodge et al., 2009). This resulted in a sequential increase of crude soluble protein concentrations after each of the addition steps (Fig. 5). At the end of the 206 hours run 89.3 ± 2.8 g/L crude soluble protein were enzymatically extracted from the PBM fed to the extraction vessel. The productivity of the system was increased from $117.3 \text{ mg} \cdot \text{L}^{-1} \text{ h}^{-1}$ to $426.6 \text{ mg} \cdot \text{L}^{-1} \text{ h}^{-1}$ (3.6 fold) with respect to a single stage cultivation strategy (non-decoupled system). Moreover, after 206 hrs about 8.7 g/L of free amino acids were released in the culture broth.

It was estimated that the amount of protein material present in the keratin-rich biowaste was about 75 % of its dry weight. This was based on the assumption that the total amino acid content in the dried PBM was equivalent to its actual crude protein content (See supplementary material). Given these facts, approximately 72.5 % of the insoluble protein material originally entrapped in the bristle structure was enzymatically recovered.

The composition of the free amino acids present in the final keratin protein hydrolysate is shown in Table 1. Among the free amino acids found in the obtained protein hydrolysate, branched-chain free amino acids (BCAAs) were particularly abundant. This was in accordance to what was recently observed by Łaba et al. (2017) when enzymatically digesting pretreated pig bristles using a concentrated cell free keratinase extract from *Bacillus cereus* PCM 2849. BCAAs, (i.e., Leucine, Isoleucine, Valine) are essential for protein synthesis (Li et al., 2009). The protein hydrolysate powder obtained after the biological degradation process exhibited a very high *in-vitro* pepsin digestibility (~ 95 %) with respect to the non-hydrolysed PBM (~ 43 %).

Table 1. Free-amino acid concentration in final hydrolysate obtained during two-stage PBM biological degradation process with *A. keratiniphila* D2.

Amino acid	Concentration (mg/L)	
	This work	Łaba et al., 2017
Ala	1039.8	54.7
Arg	101.4	119.5
Asn	No	14.7
Asp	273.0	24.2
Cys	660.9	No
Glu	1939.4	61.8
Gln	No	37.3
Gly	14.9	17.1
His	70.1	36.7
Ile	407.3	342.3
Leu	1114.4	953.0
Lys	53.7	199.4
Met	134.3	136.0
Orn	147.7	No
Phe	132.8	14.2
Pro	659.4	No
Ser	11.9	14.5
Thr	4.5	325.8
Trp	193.9	25.0
Tyr	408.8	269.3
Val	1368.0	360.6
TOT	8736.3	3006.1

3.3. Size distribution of proteins/peptides

The end hydrolysate (206 h) was subjected to SEC, in order to estimate the size distribution of protein/peptide products produced following the hydrolysis. The hydrolysate showed to contain a broad distribution of product sizes, covering the entire fractionation range of the column (2×10^6 – 1000 Da). Gel electrophoresis of fractions from the SEC run (Figure S1) showed that the mass of the products was decreasing as a function of increasing elution volumes, correlating with the results from the calibration of the column (Figure S2), which was thus used to estimate the size of the products. Using both the electrophoresis and SEC calibration results for size estimation, it was observed that the hydrolysate contained predominantly medium to small molecular mass peptides (<20 kDa), based on 280 nm absorbance monitoring. These observations were further verified by peptide concentration measurements using the BCA assay, which nicely follows the trend of the 280 nm monitoring. Free amino acid concentration in each fraction was also attempted measured and a peak was observed to co-align with the elution volume of low molecular species like vitamin B₁₂ and free amino acids (Figure 1, S2 and S3). It should be noted that the estimated amounts from the ninhydrin assay exceeded our expectations, indicating the possibility of cross reactions with other molecular species in the hydrolysate. As with results for the BCA measurements, the ninhydrin assay also indicates that a species that react with both these reagents and absorb light at 280 nm is eluting (~21 ml) after the theoretical highest exclusion volume (~20 ml). This could be due to adherence to the material in the column and similarly when free tryptophan is run over the column it elutes around 23.5 ml, which happens to co-align with the last small peak observed in the chromatogram (280 nm) and with ninhydrin. Based on these results single fractions were binned to make larger pools covering different size distributions (Table 2).

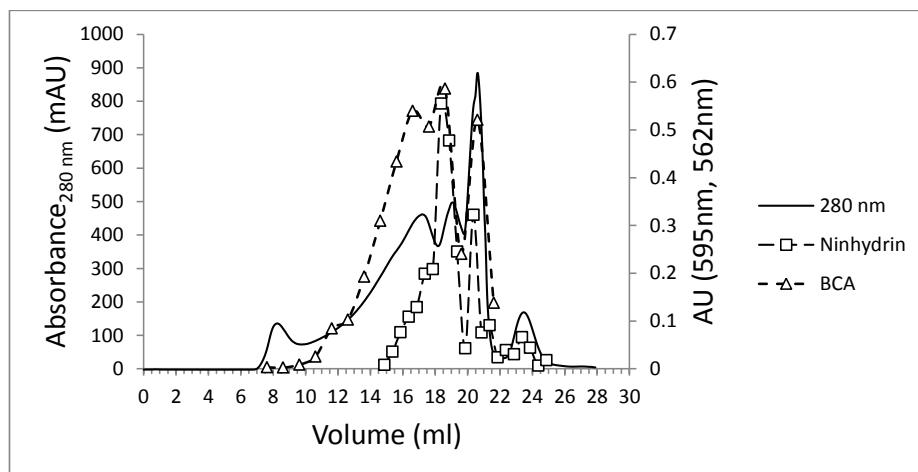


Fig. 6. Chromatogram of the SEC analysis of the end hydrolysate (206 h).

The SEC analysis was monitored by absorbance measurement at 280 nm (solid line), followed by analysis of the protein content (short dash) and free amino acids (long dash), within the collected fractions. All the analysis results indicate that the hydrolysate is fractionated according to size and that much of the protein content is found in smaller peptides.

Table 2. Summary of results from the amino acid analysis of binned fractions from the SEC analysis.

Fractions from the SEC analysis were binned according to the theoretical mass of the protein products, which is based on the calibration of the column. Groups of binned fractions were analyzed to obtain total and free amino acid content, indicating that the largest amount of amino acids were found in group C, thus in peptides/proteins with a mass starting at 5.5 kDa, down to 1.35 kDa and possibly free amino acids. The summed amount of total amino acids is 70 % of that in the sample loaded on the column (Fractionated sample).

Fraction groups	Elution ^a (ml)	Mass ^b (kDa)	Total AA (mg)	Free AA (mg)
A	7.36 – 14.86	1.6×10^3 – 23.0	0.41	-
B	14.86 – 17.36	23.0 – 5.5	1.18	-
C	17.36 – 19.86	5.5 – 1.35	2.03	0.45
D	19.86 – 22.36	1.35 – 0.33	0.24	-
E	22.36 – 24.86	0.33 – 0.79	-	-
Sum of fractions			3.86	0.45
Fractionated sample			5.48	-

^a The elution range from which fractions were binned together in the corresponding group.

^b Theoretical mass range of products within the group based on the calibration of the column.

To get a more accurate measure of the total amount of amino acids in the binned fractions, amino acid analysis was conducted on each of the fraction pools. The results verify what was already hypothesized based on the other experiments, showing that the highest concentration of amino acids were detected in fraction groups B and C with a size distribution corresponding to medium to small peptides and free amino acids (Table 1). This specific size distribution of the peptides found in the hydrolysate can prove to have a critical impact on the use of the hydrolysate as food additive. As expected the C group contains the highest amount of free amino acids, with the summed amount of free amino acids corresponding to 11.7% of the total amino acid amount. Free amino acids within the hydrolysate can also prove to be a source of amino acids that are readily metabolized by a variety of feeders.

4. Conclusions

In this work we successfully employed the filamentous bacterium *Amycolatopsis keratiniphila* D2 for the consolidate bioprocessing of pretreated pig bristles to keratin protein hydrolysates. Indeed, by means of the developed two-stage biological recovery process extraction yield, titre and volumetric productivity of crude soluble protein were significantly improved. It was additionally shown that, the largest fractions detected in the final keratin protein hydrolysate were those consisting of small peptides and free amino acids. This together with the high *in vitro* digestibility of the obtained product shows the great potential of this integrated production strategy in obtaining protein supplements for animal feed with improved nutritional value.

Acknowledgments

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Supporting Figures:

S1:

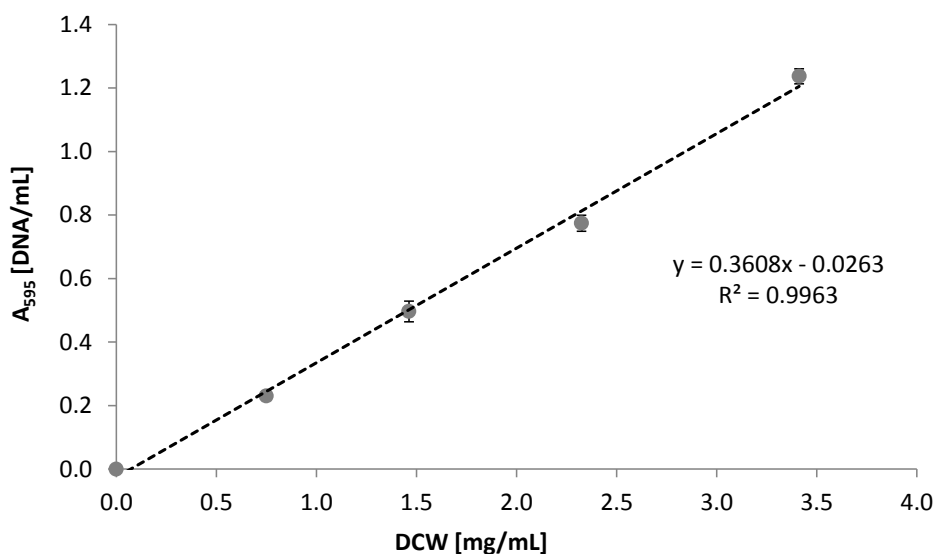


Figure S1: Calibration curve correlating *Amycolatopsis keratiniphila* D2 dry cellular weight (DCW) to the absorbance measured at 595 nm and obtained employing the simplified diphenylamine DNA quantification method. In order to obtain a reliable and accurate quantification of microbial cell growth in a cultivation medium containing solid keratin particles it was chosen to employ the DNA extraction and quantification method developed by Zhao et al. (2013). The diphenylamine colorimetric method to quantify microbial DNA showed a very good correlation with the measurement of cell density obtained with a standard cell dry weight method.

S2:

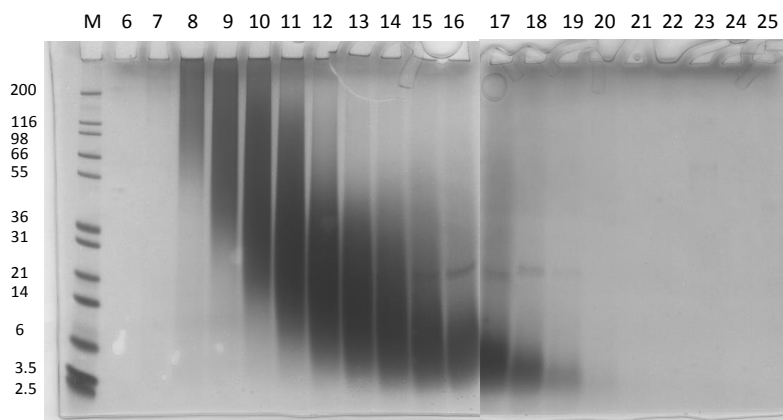


Figure S2: Gel electrophoresis of fractions from SEC analysis of hydrolysate. Fractions from the SEC analysis were run on a Nu-PAGE gel to verify that the products within the hydrolysate were distributed within the fractions according to size. Numbers above lanes correspond to the elution volume (ml) of the fraction. The marker (M) is the Mark 12[®] protein ladder and numbers on the left side of the gel correspond to the molecular masses (kDa) of the protein standards.

S3:

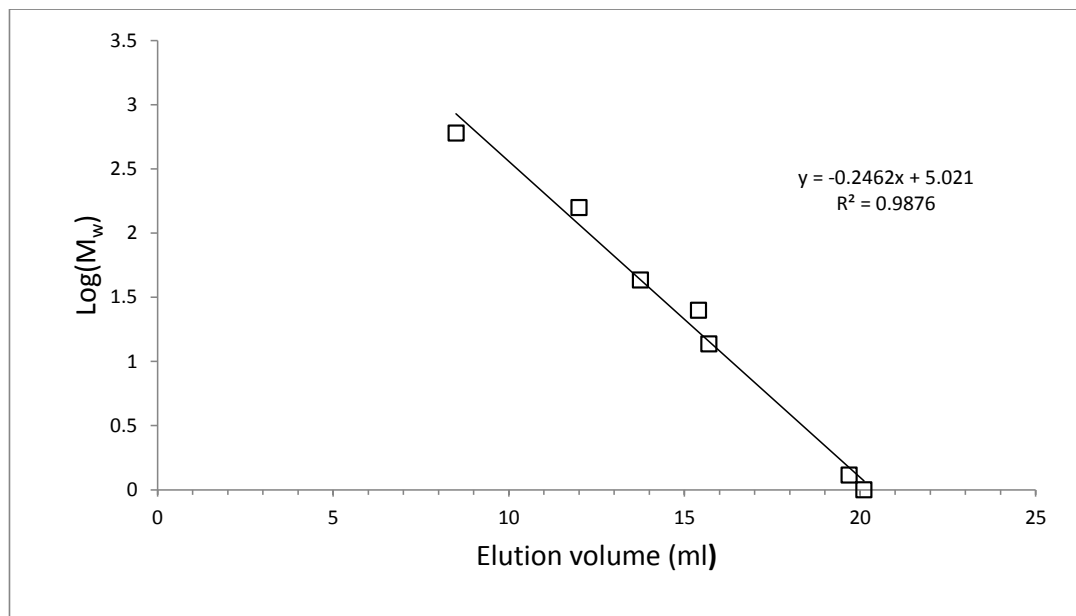


Figure S3: Calibration of Superose 12 column. The Superose 12 column was calibrated using a range of proteins specified in the Materials and Methods section. A linear model was fitted to the data and used to estimate the sizes of the products in the hydrolysate. The formula obtained from the fit as well as the R^2 is shown within the plotting area.

S4:

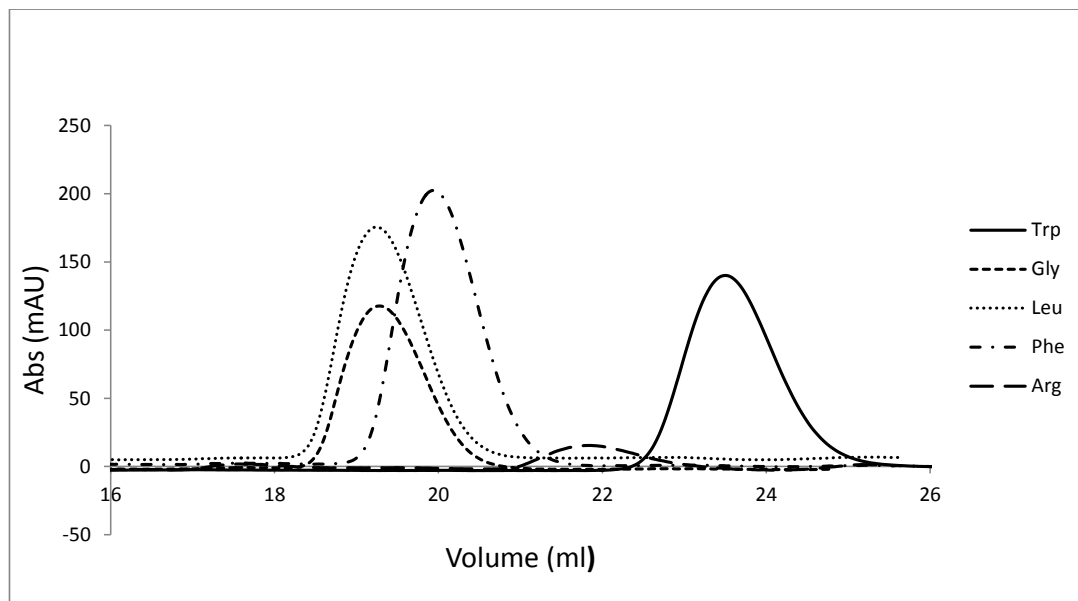


Figure S4: SEC of free amino acids. In order to know when free amino acids would elute from the Superose 12 column Trp, Gly, Leu, Phe and Arg were run on the column. It is observed that Gly, Leu and Phe elute as expected by molecule according to their size, while Arg and Trp elute much later. Arg and Trp are thus suspected to adhere to the column material and elute later than expected.

Chapter 4

Bioconversion of pretreated porcine bristles into protein-rich hydrolysates for aquafeed by the keratinolytic actinobacterium *Amycolatopsis keratiniphila* D2.

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Abstract

Keratin-rich residual biomass such as porcine bristles constitutes an abundant hair-type keratinous by-product and one of the most recalcitrant, and hard-to-degrade keratin waste materials. At this point in time, the effective reutilization of this keratinous waste stream still represents an unsolved technological challenge, not to mention the serious environmental risks and health hazards involved in the poor management of this solid agro-industrial residue. In this study, a potent keratinolytic actinobacterium, namely *Amycolatopsis keratiniphila* D2, was first cultivated in a keratin mineral medium in order to produce enzymes with proteolytic and keratinolytic activity. In the following stage, the obtained cell-free crude keratinases extract was employed to direct the biodegradation of pretreated pig bristles at high solid loading (15 % w/w). To this end, with the aim of maximizing crude protein extraction, two different fed-batch strategies were tested. In both strategies the solid substrate was split into four equal aliquots (25:25:25:25 ratio) which were then added to the vessel after regular intervals of 24 hours each. Moreover, in the first strategy (StgA) also the crude enzyme preparation was split into four aliquots (same 25:25:25:25 ratio) and fed stepwise to the vessel together with the solids, while in the case of the second strategy (StgB) all of the crude enzyme preparation was added from the start. The first methodology resulted in an overall accumulation of 69.4

g/L of crude soluble proteins with a 53.0% substrate loss while, the second approach, allowed the release of 73.2 g/L of crude soluble proteins with a 58.1% substrate loss. In addition, the nutritional quality of the obtained bristle protein hydrolysates (BPH_S) was characterized by means of *in vitro* tests in order to assess the potential of the obtained protein-rich digestates for use as fishmeal replacement in fish feed formulations. For instance, BPH_A and BPH_B displayed a 1.8 and 2.1 fold increase respectively in their biological values (BVs) with respect to the non-hydrolysed pretreated substrate. Moreover, size exclusion chromatography (SEC) was performed on both BPH_A and BPH_B revealing the presence of different peptides in each of the two products. Additionally, it was demonstrated that both BPH_A and BPH_B were also rich in free amino acids containing respectively 11.4 and 8.8 grams of them per 100 grams of dry protein feed. Therefore, it is possible to suggest that the soluble proteins, peptides and free amino acids recovered during the enzymatic extraction process could be used as a viable alternative protein source in aqua feed.

Abbreviations

PBM porcine bristle meal

StgA feeding strategy A

StgB feeding strategy B

BPH_A bristle protein hydrolysate A

BPH_B bristle protein hydrolysate B

PDCAAS digestibility-corrected amino acid scoring

BV biological value

SEC size exclusion chromatography

1. Introduction

Keratins are fibrous structural proteins, insoluble in water and recalcitrant to enzymatic lysis with common proteases such as papain, trypsin and pepsin (Korniłowicz-Kowalska and Bohacz, 2011;

Daroit and Brandelli, 2014). The main structural element of a keratinous protein consists of a super-coiled motif of α -helices (α -keratins, 40-60 kDa) or β -sheets (β -keratins, 10 kDa) that is arranged in a tightly packed secondary structure and rendered stable by the action of a high number of disulphide bridges together also with hydrogen bonds and hydrophobic interactions (Kothari et al., 2017). Keratins are found in the epidermis and epidermal appendages of vertebrates namely, skin remains, scales, nails, feathers, beaks, horns, hooves, bristles, wool, etc. Keratinaceous biomass is a naturally occurring polymeric material, and after cellulose and chitin constitutes the third most abundant biopolymer present in nature (Lange et al., 2016). Among others, porcine bristles can be regarded as a particularly underestimated and therefore underexploited keratin-rich by-product which is generated in slaughterhouses and meat processing plants and represents a serious environmental problem. As a matter of fact, the exceptional sturdiness of pig bristles and other hair-type keratinic appendages (including for example wool) hindered the effective management of this specific category of solid waste stream (Fakhfakh et al., 2013; Fang et al., 2013a; Łaba et al., 2015a; Łaba et al., 2017). In this respect, since the protein content of porcine bristle can be even more than 80% of its dry weight, and considering that in 2014 about 250 million pigs were produced and slaughtered in the EU (EUROSTAT, 2016), these figures mean that a potential of 60 k-tons dry proteins could be recovered from this solid waste and could be employed, for example, as a renewable and sustainable source of animal feed proteins. For instance, the conventional method employed for converting pig bristles into a more digestible dietary protein consists of the hydrothermal cooking with or without acid or alkali pretreatment (Latshaw et al., 1994; Chojnacka et al., 2011). Nonetheless, the porcine bristle meal (PBM) obtained through this process route is usually lacking sufficient essential amino acids, such as methionine, histidine, tryptophan and lysine and has still a relatively low digestibility (Hertrampf and Piedad-Pascual, 2000b; Bertsch and Coello, 2005). Consequently, it would be of great interest if one could develop milder biotechnological methods, which

could effectively recover soluble proteins, peptides and amino acids from the hair-type keratinous waste by keeping the extracted nutrients intact and even augmenting the nutritional quality of the obtained keratin protein hydrolysates (Sahoo et al., 2017). Some naturally occurring bacteria, actinomycetes and keratinophilic fungi possess the ability to employ keratin as their unique source of C, N, S and energy. That is because they are able to synthesize keratin-specific extracellular proteolytic enzymes, viz. keratinases, which are capable of effectively degrading and hydrolysing keratin-rich waste materials (Verma et al., 2017). Keratinases are usually encountered within both classes of serine and metallo-endoproteases, exhibiting a typical average size in the range of about 30–50 kDa (Brandelli et al., 2010; Sharma and Devi, 2018). Enzymatic digestion of keratinous by-products with cell-free crude keratinase extract has emerged as one of the possible approaches for the bioconversion of keratinaceous biowaste into nutritionally valuable protein hydrolysates. In particular, the development of an enzymatic process utilizing keratinolytic proteases for the biotransformation of recalcitrant hair-type keratinous by-products into a dry keratin protein hydrolysate which could then be employed as a fish meal replacement could constitute an interesting biotechnological route to valorise residual keratinous biomass. In fact, dietary protein is the principal and most costly ingredient contained in aquafeed formulations and one of the main factors affecting the overall productivity of an aquatic farm (Wilson, 2002; Lall and Dumas, 2015). In order to provide a well-balanced diet, the nutritional importance of each of the amino acids present within formulated aquafeeds must be considered. To this end, ten amino acids are classified as essential (i.e., considered as indispensable) in promoting balanced growth, development and well-being of farmed fishes. The amino acids belonging to this group are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. These amino acids either cannot be synthesized or are inadequately synthesized by aquatic animals and for this reason, they must be incorporated into the diet. Furthermore, cysteine and tyrosine, which are synthesized in the body of the fish

starting from methionine and phenylalanine respectively, must be also included in the administered diet (Li et al., 2009; Lall and Dumas, 2015). Furthermore, another seven amino acids (alanine, aspartic acid, asparagine, glutamic acid, glutamine, proline, and serine) are categorized as nonessential (i.e., considered dispensable) for fish because their organism is capable of synthesizing them from simpler precursors. In general, with the objective of fully satisfying the dietary requirements of the fish both in terms of protein synthesis efficiency and growth performance, essential amino acids must be readily available inside the feed with no limitations. Moreover, a dietary protein must supply a sufficient amount of nonessential amino acids or contain enough nitrogen of amino acid origin to enable the aquatic organism to synthesize them. As demand for seafood globally continues to grow, most of the additional supply is coming from fish farms. The reason behind this trend is that, for the past two decades, the world's wild fisheries have struggled to increase their output mainly owing to natural fish stocks depletion from overfishing, which is why nowadays half of the global seafood available on the market comes from aquafarms. As a result, there is a constantly rising demand for dietary protein, mainly in the form of fish meal, to meet the growing need of the fish farm industry. Fishmeal is made for the most part from wild-caught small marine fish and is generally regarded as the most nutritionally suitable dietary protein for aquatic animals and therefore it has been, during the past decades, the most largely employed source of amino acids in aquaculture. Nevertheless, in recent years, overfishing has also become one of the main drivers behind the stagnation in the supply of wild-caught fish for feed and the consequent detrimental effect on the fishmeal market price which has quadrupled over the past two decades. As a matter of fact, overexploitation of aquatic resources has meant that at the moment much of the protein included in feed comes from plant sources, which is most commonly soybean. However, a diet including too much soy proteins both in the form of soybean meal or soy proteins concentrate could have a negative effect on feed intake and fish growth because it contains allergenic proteins and other anti-

nutritional factors which are especially unwanted when feeding juvenile and sub-adult fish specimens (Hou et al., 2017). That is why the feed industry is constantly looking at developing new sources of dietary proteins for the aquaculture which can be both a nutritionally balanced source of amino acids and represent an affordable alternative to fish meal and soybeans. Therefore, in the present work a biotechnological process for the production of bristle protein hydrolysate consisting of two physically separated stages was developed. Moreover, based on the characterization of the nutritional value of the obtained keratin hydrolysates, it was demonstrated that enzymatically hydrolysed porcine bristle meal could be considered as a potential protein-rich alternative for use as fishmeal replacer in fish feed formulations.

2. Materials and Methods

2.1. Porcine bristle meal

The slaughterhouse by-product used as C, N and energy source for microbial growth and employed as well to prepare azokeratin for activity assays was kindly supplied by DAKA SARIA Group A/S (Løsning, Denmark). The keratinous residual material consisted mainly of porcine bristles, which were chopped, thermally pretreated (150 °C, 6 bars, 20 min), dried and finally crushed into smaller particles.

2.2. Chemicals and gases

All chemicals employed in this study were of analytical grade and were purchased from Sigma Aldrich ApS (Brøndby, Denmark); gases were supplied by AGA A/S (Copenhagen, Denmark).

2.3. Microorganism and media composition

The strain *Amycolatopsis keratiniphila* D2 (DSM 44409) was obtained from DSMZ (Braunschweig, Germany). Seed culture glycerol stocks were prepared and stored at -80 °C prior to use. *A. keratiniphila* D2 was cultivated in mineral keratin (Basal) medium formulated according to the fol-

lowing composition (g/L): 0.75 NaCl, 1.75 K₂HPO₄, 0.25 MgSO₄·7H₂O, 0.055 CaCl₂, 0.010 FeSO₄·7H₂O, 0.005 ZnSO₄·7H₂O, 10.53 (1 % w/w) PBM powder. The medium was sterilized at 121 °C for 20 min before use.

2.4. Inocula preparation

Inocula were prepared by propagating frozen microbial cell stocks of *A. keratiniphila* D2 in Erlenmeyer flasks (500 mL) containing 100 mL of GYM medium (4.0 g/L glucose, 4.0 g/L yeast extract, 10.0 g/L malt extract); the initial pH of the culture broth was adjusted to 7.2 before autoclaving. Each flask was inoculated with 1 mL of a glycerol stock (20 % v/v) of bacterial cells and incubated on an orbital shaker (200 rpm) at 28 °C for 48 hours. Microbial cells were harvested by centrifugation (4000 rpm for 20 min at 4°C), washed twice in sterile phosphate buffer saline (PBS) solution (15 mM NaCl, pH 7.2) and again resuspended in an appropriate volume of PBS solution.

2.5. Enzyme assay

Crude keratinase activity was assayed on supernatant obtained after centrifugation (12000 × g, 5 min) of the fermentation broth with azokeratin as a substrate by the following method. The reaction mixture contained 200 µL of enzyme preparation and 1400 µL of 10 g azokeratin L⁻¹ in 50 mM tris buffer, pH 8.0. The mixture was incubated for 15 min at 50 °C; the reaction was stopped by the addition of 1600 µL Trichloroacetic acid (TCA) to a final concentration of 100 g/L. After centrifugation at 10,000 × g for 5 min, the absorbance of the supernatant was determined at 440 nm. One unit of enzyme activity was the amount of enzyme that caused a change of absorbance of 0.01 units at 440 nm after 15 min incubation at 50 °C. Caseinolytic activity was assayed by a similar protocol using azocasein as substrate.

2.6. Crude soluble proteins determination

Soluble protein concentration was determined by the bicinchoninic acid (BCA) assay (Smith et al., 1985) employing bovine serum albumin (BSA) as protein standard (Pierce™ BCA Protein Assay Kit, Thermo Scientific).

2.7. Size exclusion chromatography of bristle protein hydrolysates

Hydrolysate from the end of the hydrolysis process (144 h) was used for size analysis. The sample was prepared by filtration through a 0.22 µm filter (Minisart NML, Sartorius™), followed by centrifugation (15 min, 10,000 g). The supernatant was diluted ten times into the running buffer (10 mM $\text{NH}_4^+(\text{CH}_3\text{COO}^-)$, 30% acetonitrile in water at pH 9 and then centrifuged again (15 min, 10,000 g). A Superose® 12 10/300 column was equilibrated with running buffer (10 mM $\text{NH}_4^+(\text{CH}_3\text{COO}^-)$, 30% acetonitrile in water at pH 9 and then 1 ml of the diluted sample was loaded inside the column. The flow rate was set to 1 ml/min, the separation was monitored by measurement of absorbance at 280 nm and the size separated sample was collected in 0.5 mL fractions. Calibration of the Superose® 12 10/300 column was performed by two consecutive runs of a mixture of calibration samples. The first run consisted of Aldolase (36 kDa), Chymotrypsinogen (25.6 kDa) and vitamin B¹² (1.3 kDa). The second run consisted of Thyroglobulin (660 kDa), Ovalbumin (66 kDa) and Ribonuclease (13.7 kDa). Furthermore, a measurable change in conductivity was considered as an indicator for elution of the smallest possible molecules. The calibration was performed in a different buffer than the one used for the sample (10 mM NaPi, 500 mM NaCl at pH 7.5). The molecular mass of the calibration molecules (kDa) was Log_{10} transformed and plotted as a function of elution volume (K_e), after that a linear model was fitted to the data ($\text{Log}_{10}(\text{mass}) = a * K_e + b$). The column was further calibrated by running free amino acids (Gly, Leu, Arg, Phe and Trp) over the column to test whether these would adhere to the column material when using the running buffer used for the sample runs.

2.8. Amino acid analysis and *in vitro* digestibility of bristle protein hydrolysate powder

Freeze dried samples were shipped to Eurofins Agrosience Services A/S, Middelfart, Denmark, <https://www.eurofins.com/agrosienceservices> for total nitrogen, crude fat, crude ash, amino acid analysis and for performing an *in vitro* pepsin digestibility test. Samples were both analyzed for total amounts of amino acid (acid hydrolysis of samples) and free amino acid content (no acid hydrolysis of samples).

2.9. Nutritional parameters of bristle protein hydrolysates

Amino acid composition and *in vitro* digestibility of PBM and BPH_A and BPH_B, were employed to evaluate the nutritional value of the original substrate and of the two bristle protein hydrolysates. The protein digestibility-corrected amino acid scoring (PDCAAS) was determined according to Abdul-Hamid et al. (2002). Predicted biological value (BV) was calculated utilizing the following equation (Morup and Olesen, 1976):

$$BV = 10^{2.15} \times q_{Lys}^{0.41} \times q_{Phe+Tyr}^{0.60} \times q_{Met+Cys}^{0.77} \times q_{Thr}^{2.4} \times q_{Trp}^{0.21}$$

where $q = a_i(\text{sample})/a_i(\text{reference})$ for $a_i \text{ sample} \leq a_i \text{ reference}$

$q = a_i(\text{reference})/a_i(\text{sample})$ for $a_i \text{ sample} \geq a_i \text{ reference}$

$a_i = (\text{mg of the amino acid})/(\text{g of total essential amino acid})$

2.10. Production of keratinolytic enzymes in laboratory scale fermenter

Batch submerged cultivation of the filamentous bacterium *A. keratiniphila* D2 were carried out in two identical 5-L bioreactors (BIOSTAT® B, Sartorius), with a 4.5-L working volume. Both fermenters were equipped with two 6-bladed Rushton impellers. Cultivation parameters were optimized in order to maximize keratinolytic enzyme secretion: the initial pH of the keratin basal medium was adjusted to 7.2; the temperature was set at 28 °C; fully aerobic conditions (dissolved oxygen concentration $\geq 30\%$) were ensured by flowing 1 vvm of air throughout the vessel; stirring was

kept at a constant rate of 500 rpm. Fermenters were inoculated with 2.5 % (v/v) of fresh inoculum ($\approx 0.35 \text{ mg}_{\text{cdw}}/\text{ml}$) and run under the previously described conditions for up to 84 h. During the course of the cultivation of *A. keratiniphila* D2 in a keratinous waste containing medium an increase of pH was observed. Once a value of 8.0 was reached, in order to avoid a further increase of alkalinity in the culture broth, the pH was stabilized around a set point value of 8.0 and then controlled by adding 2M H_2SO_4 with a peristaltic pump. At the end of the keratinases production process, the supernatant was separated from the residual non-hydrolysed keratin and spent microbial mass by centrifugation at 4000 rpm for 40 min (Heraeus™ Multifuge™ X3R, Thermo Fisher Scientific). In addition vacuum filtration was employed to further remove small insoluble particles from the free-cell crude keratinase extract down to an average particle size of 12-15 μm (Sartorius™ Grade 288 Qualitative Filter Papers Disc). Afterwards, the keratinase solution free of solids was up-concentrated about 9 fold using a laboratory cross-flow ultrafiltration unit with a molecular cut-off size of 2 kDa (Vivaflow 200, Sartorius). Finally, the concentrated cell-free crude keratinase solution was divided into aliquots and frozen at -80°C to be stored before use.

2.11. Enzymatic hydrolysis of pretreated pig bristles with cell-free keratinase extract

Enzymatic hydrolysis experiments of porcine bristle meal with cell-free crude keratinase extract were carried out in a 3-L bench-scale bioreactor (Applikon, Delft, The Netherlands) with a 1.5-L working volume and equipped with two 6-bladed Rushton impellers. Process parameters were optimized in order to maximize both the rate of keratin degradation and the extent of crude protein extraction and solubilisation as well: the temperature was maintained at 50°C ; the stirring rate was fixed at 800 rpm; the automatic addition of a 6 M Na_2OH solution prevented acidification of the hydrolysate and kept its pH around a set point value of 8.0. The hydrolysis process was carried out for a total of 6 h. At the end of the degradation process, the protein hydrolysate was separated from the residual non-solubilized keratin by centrifugation at 4000 rpm for 40 min (Heraeus™ Multi-

fuge™ X3R, Thermo Fisher Scientific). In addition vacuum filtration was employed to further remove small insoluble particles from the protein digest down to an average particle size of 12-15 µm (Sartorius™ Grade 288 Qualitative Filter Papers Disc). Finally the bristle protein hydrolysate was lyophilised in a CoolSafe™ 4 L freeze dryer (LaboGene, Denmark) in order to obtain a dry powder.

2.12. Statistical analysis

Experimental data were analysed for statistical significance ($p < 0.05$) using one-way ANOVA, followed by Tukey's honestly significantly different (HSD) test.

3. Results and discussion

3.1.Stage 1: aerobic batch production of proteolytic and keratinolytic enzymes.

The first stage of the developed two-step biotechnological process for the production of bristle protein hydrolysates consisted of obtaining enzymes with proteolytic and keratinolytic activity from *A. keratiniphila* D2 in a laboratory scale fermenter by using PBM as keratinase inducer. To this end, in order to achieve optimal microbial growth under fully aerobic conditions and as a result maximum rate of both extracellular proteolytic and keratinolytic enzymes production, the cultivation temperature was maintained at 28 °C and 1% w/w pretreated porcine bristles were utilized as the only source of C, N, S and energy for growth. In addition, the initial pH was adjusted to 7.2 and controlled around a set point value of 8.0 by acid addition. At the same time, the aeration rate was kept at 1.0 vvm to provide fully aerobic conditions ($DO \geq 30\%$), and meanwhile the agitation rate was maintained constant at 500 rpm. As a result, during the batch cultivation of *A. keratiniphila* D2 in a 3-L aerobic fermenter maximum proteolytic activity was observed after 60 hours (359 ± 32.9 kU/L) while the highest level of keratinase accumulated in the culture broth was reached after 84 hours of aerobic submerged cultivation (154 ± 11.4 kU/L) (Fig. 1). Even though azocaseinolytic activity detected in the supernatant was considerably higher when compared to the azokeratinolytic activity, both enzymatic activities appeared to be related. This was also noticed for example by Daroit et al.,

(2009) when studying the production of extracellular proteases in 1% (w/w) feather mineral medium with *Bacillus* sp. strain P45. For instance, Fontoura et al., (2014) showed that the keratin-degrading strain *Chryseobacterium* sp. kr6 secreted the maximum amount of active proteolytic enzymes in the culture broth after 24 hours of cultivation on 75 g/L thermally denatured chicken feathers. Proteolytic activity determined on azocasein was about 175 U/mL, even if it then decreased slightly, and was lower than the one measured in this work. Moreover, Lemes et al. (2016) detected both azocaseinolytic and azokeratinolytic activities in supernatant produced with the keratinophilic bacterium *Bacillus* sp. 45 grown on different agro-industrial waste substrates. Maximum proteolytic activity (circa 1307 U/mL) was found in 32 hours old supernatants obtained by microbial growth on residual yeast biomass from *K. marxianus*, which is significantly higher than that measured in this study. Regarding the production of enzymes showing keratinolytic activity the highest amount (89 ± 2.0 kU/L) was accumulated after 32 hours of submerged cultivation on reused feather meal as substrate.

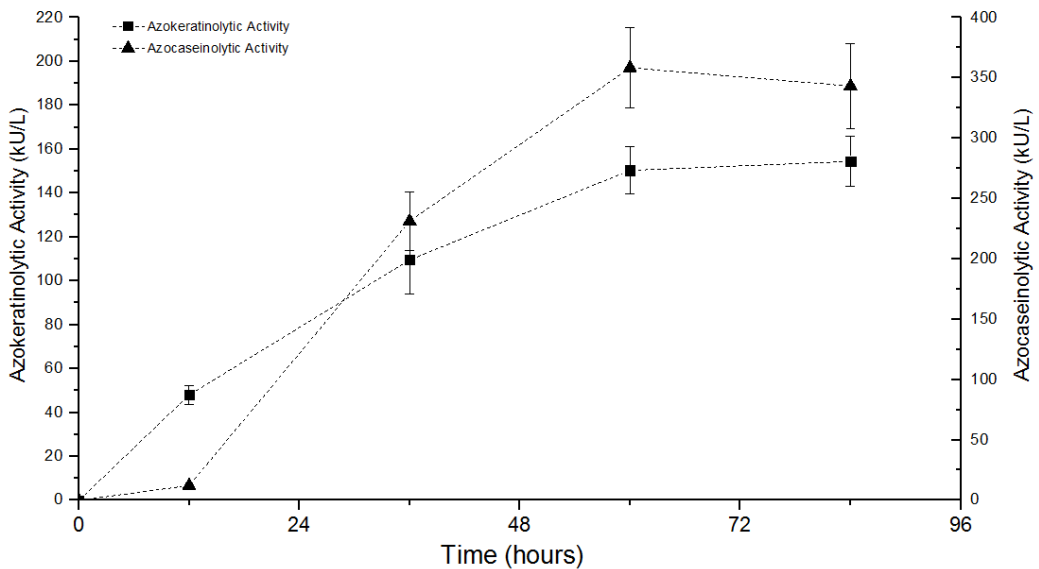


Fig.1. Extracellular proteases and keratinases production by *Amycolatopsis keratiniphila* D2 as a function of cultivation time.

On the contrary, this value is appreciably lower when confronted with what has been observed in this study. This could explain the major keratinolytic power displayed by *A. keratiniphila* towards a more recalcitrant substrate than feather (i.e., porcine bristles) when compared with *Bacillus* sp. strain 45. In fact, this strain was unable to degrade human hair which is another type of keratinous waste material also containing a large fraction of β -keratin within its structure (Daroit et al., 2009). Considering that hog hair is an inexpensive, abundant and readily available, agro-industrial by-product it is reasonable to consider its utilization by keratinophilic microorganisms both as cheap substrate for growth and proteinase inducer. Consequently, the use of keratin-degrading bacteria, actinomycetes and fungi serves as a feasible and cost-effective method for the biotechnological production of enzymes with both proteolytic and keratinolytic properties. The keratinolytic actinobacterium *A. keratiniphila* D2 employed in this work resulted to be a potent proteinase and keratinase producer as we have demonstrated that this strain was capable of biodegrading efficiently hair-type α -keratinaceous waste material which is known for being extremely recalcitrant to the hydrolytic action of both proteolytic and keratinolytic enzymes.

3.2.Stage 2: biodegradation of pretreated pig bristles with cell-free crude enzyme extract

Water is the essential medium where keratins are first extracted and solubilized than decomposed into polypeptides, which in turn are finally hydrolyzed by cleavage of peptide bonds within amino acid chains. In fact, water is the key solvent for enzymes to diffuse in and for products to diffuse away from reaction sites. Additionally, the presence of free water within the slurry confers lubricity to the particulate system reducing viscosity and decreasing the power input that is required during mixing to obtain the desired shear rate (Palmqvist and Lidén, 2012). When enzymatic degradation is performed at high insoluble solid loadings the lack of available water in the reactor might give rise to rheological challenges, cause inadequate mixing, reduce mass and heat transfer efficiency, and increase the concentration of inhibitory compounds present within the mixture, resulting in a drastic

reduction of the yield of extraction of proteins and their following hydrolysis (Geng, et al., 2015). Nevertheless, the use of high-solids loadings during enzymatic hydrolysis of keratinaceous by-products presents several advantages such as the possibility to employ smaller equipment, with the consequent reduction of energy input for heating the vessel, decrease hydraulic loadings, and obtain larger titres in crude soluble protein at the end of the process (Modenbach and Nokes, 2013). During a fed-batch operation, substrates and/or enzymes are supplied stepwise to the extraction tank by following an established sequence. For the case of lignocellulosic biomass it has already been demonstrated that, when using a fed-batch strategy, due to a lower initial viscosity which minimizes diffusion limitations, it is possible to achieve a higher initial rate of hydrolysis and, moreover, enough time is provided for the slurry to liquefy before the fresh particulate material is added to the vessel (Hodge et al., 2009). Among the most important factors that must be considered, the rate of enzymatic hydrolysis, the final concentration and extraction yield of crude soluble proteins are all critical parameters to be accounted for when evaluating the techno-economic feasibility of a commercial process for the recovery of proteins from a particular keratin-rich waste material. During the second stage of the process, biodegradation of pretreated porcine bristles for the production of keratin protein hydrolysate was carried out in a 3-L laboratory scale stirred tank bioreactor. The cell-free crude keratinase extract produced by *A. keratiniphila* D2 during the first stage of the process was employed in the second phase for the enzymatic hydrolysis of the thermally denatured hog hairs. At this point in time, the bioprocess conditions were chosen with the objective of maximizing the rate of hydrolysis of the recalcitrant keratinous by-product. Therefore, in the second part, the temperature was maintained at a constant value of 50 °C while pH was stabilized around a set point equal to 8.0. Additionally, no air was supplied to the hydrolysis vessel, while the agitation rate was kept at 800 rpm. Solids were added stepwise at the hydrolysis times of 0 (92.7 g WIS in 1.85 L), 24 (185.3 g WIS in 1.90 L), 48 (277.8 g WIS in 1.95 L) and 72 h (370.4 g WIS in 2.00 L)

to reach a total insoluble solids loading of 15% (w/w) (the solid substrate was split into fractions of equal size, i.e., 25:25:25:25%). The addition of the crude enzyme cocktail was performed in two different ways. For StrA, enzymes were loaded into the reaction vessel in order to maintain a constant enzymes/substrate ratio of about 729 U/g-substrate during the course of keratin decomposition and hydrolysis, while in the case of StrB the whole quantity of biocatalyst was added directly from the start. As it can be graphically seen from the analysis of Fig.2, it was found that 53.0% ($\pm 1.5\%$) and 58.1% ($\pm 4.4\%$) of the initial porcine bristle mass was extracted at the end of 6 days of enzymatic biodegradation in case of StrA and StrB respectively. The hydrolysis levels of thermally pretreated pig bristle obtained in the current study were higher than those reported for *Bacillus cereus* B5esz by Łabaet al., (2015a). In their work on thermo-chemically treated hog bristles they demonstrated that it was possible to enzymatically digest, with a concentrated free-cell crude keratinase extract, after 24 hours, 24.7% of the hair-type pretreated keratinous substrate. Microbial cells, on the other hand, were able to microbiologically degrade 30.7% of the thermo-chemically treated waste bristles within 10 days of cultivation. The amount of crude soluble protein assayed in the final protein digestate which was released after 6 days of enzymatic hydrolysis from the keratinaceous thermally denatured substrate was 69.4 ($\pm 3.8\%$) and 73.2 ($\pm 1.8\%$) g/L when considering BPH_A and BPH_B respectively. The measured values were considerably larger than the concentration of crude soluble proteins obtained by Łabaet al. (2015a). Specifically, 1.8 g/L of soluble proteins were accumulated within 24 hours when thermo-chemically treated hog hairs were enzymatically digested with a concentrated free-cell crude keratinase extract obtained with *Bacillus cereus* B5esz.

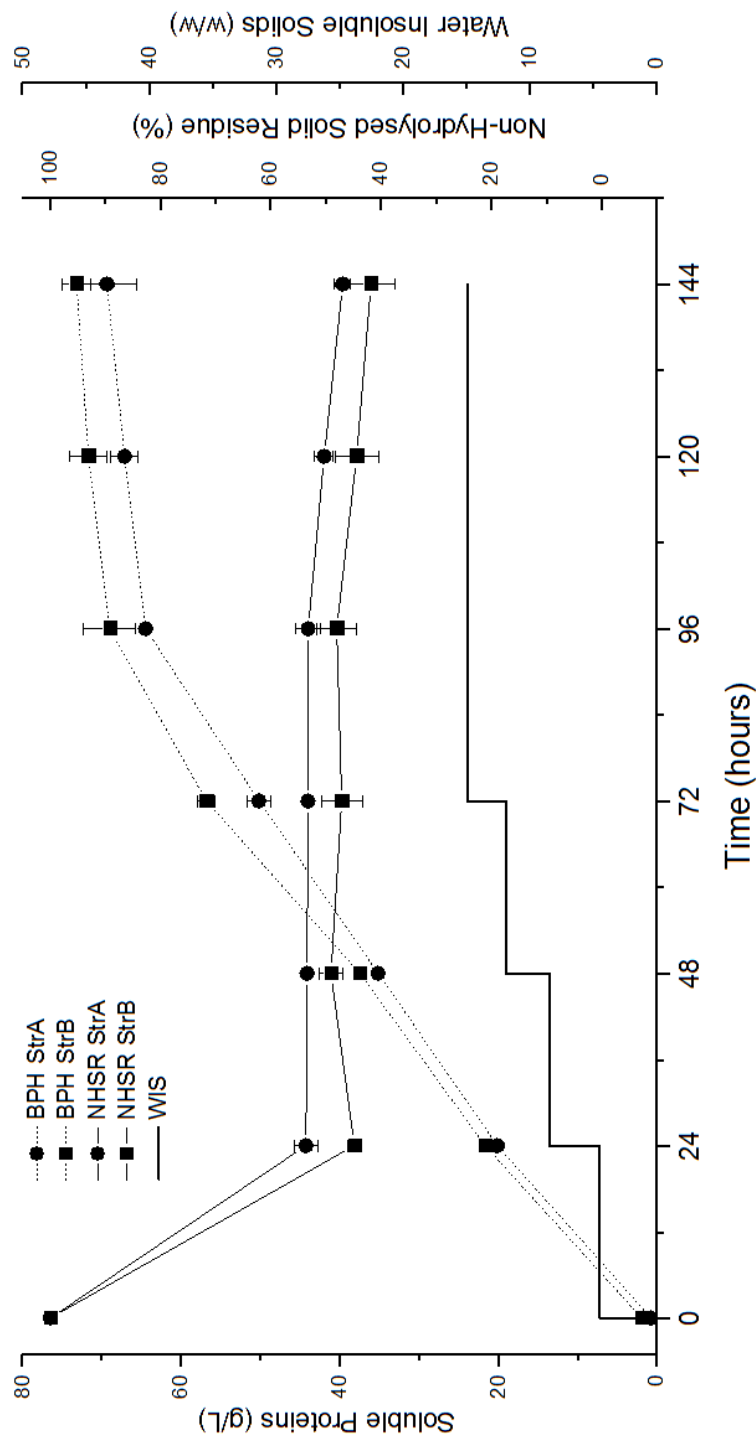


Fig.2. Time course of PBM enzymatic degradation process in a 3-L bench-scale bioreactor. Soluble proteins (dotted lines) StrA (●) and StrB (●); Residual solids (solid lines) StrA (■) and StrB (■) and water insoluble solids (—).

In analogy to what was observed by Geng et al. (2015) in their study on the high-solids (15 % w/w) enzymatic hydrolysis of dilute-acid pretreated corn stover our results also suggested that the addition of the entire quantity of enzyme to the reaction tank from the begin is more effective than its stepwise addition in proportion to the solid insoluble material concurrently fed to the bioreactor.

3.3.Size distribution of protein/peptides

SEC was performed on both BPH_A and BPH_B end products in order to gain a qualitative insight of the size distribution of protein/peptide crude extract prepared following the keratinolytic hydrolysis of the keratin-rich waste substrate. Each sample was centrifuged (15 min, 10000 g) and filtered with a 0.22 µm syringe filter before being eluted through the column. Both BPHs were characterized by a broad molecular size distribution, covering the entire range of molecular weights of the column ($2 \cdot 10^6$ - 1000 Da). Each bristle hydrolysate sample displayed three discrete curve shoulders (at 16.59 min - 338.10 mAU, 18.93 min - 287.05 mAU and 20.22 min - 812.12 mAU for BPH_A; at 16.66 min - 361.65 mAU, 18.88 min - 305.27 mAU and 20.23 min - 804.71 mAU for BPH_B) and two distinct peaks (at 7.92 min - 103.19 mAU and 22.77 min - 219.56 mAU for BPH_A; at 7.90 min - 159.94 mAU and 22.82 min - 227.64 mAU for BPH_B) which clearly revealed the presence of different peptides. Interestingly, no significant difference was observed in between the two obtained SEC spectra, indicating that the molecular mass distributions of the two keratin protein hydrolysate products were almost identical. For instance, the molecular size distribution of peptides resulting from the keratinolytic hydrolysis of thermally denatured bristles could be influenced by the specificity of the proteolytic enzymes employed (Kristinsson and Rasco, 2000; Bougatef et al., 2010). Indeed, the appearance of few predominant peaks would suggest that the cleavage of the keratinous substrate had occurred at very specific sites (Wang et al., 2003). Therefore, it could be speculated that molecular size distribution of the two products did not differ since the exact same keratin-

specific proteolytic system was employed for the preparation of both BPHs, with the only difference that the biocatalyst was fed to the enzymatic reactor in two different ways.

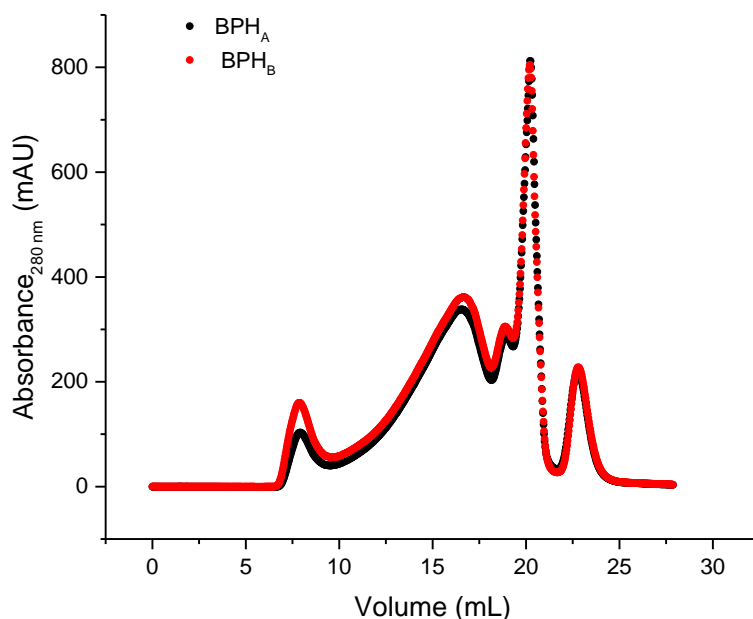


Fig.3. Chromatogram of the SEC analysis of the end products BPH_A and BPH_B (144 h).

3.4.Nutritional characterization of bristle protein hydrolysates

Among the nonessential amino acids occurring in native pig bristles glutamic acid, aspartic acid and serine are the most abundant, while within the group of the conditionally essential both cysteine and proline are plentiful. Moreover, when considering the essential amino acids, arginine and leucine are the ones which are present in the largest quantities. Besides, hog hairs are normally deficient in three important essential amino acids that are histidine, methionine and tryptophan (Graham et al., 1949; Morán et al., 1967; Esteban et al., 2010). One of the main differences that can be observed in terms of amino acid composition when comparing a commercial porcine bristle meal, normally ob-

tained by steam pressure-cooking, with respect to the untreated pig hairs is that a certain fraction of some of the essential amino acids such as cysteine, methionine and lysine is lost (Gehle et al., 1967; Wang and Parsons, 1997), which is also the case for chicken feather meals with respect to native chicken feathers (Papadopoulos 1985). In particular, cysteine and lysine are generally converted into lanthionine and lysinoalanine respectively, which are two nonproteinogenic and therefore non-nutritive amino acids (Latshaw et al., 1994; Chojnacka et al., 2011). In addition to the lower nutritional value when the hog hairs are treated through hydrothermal methods, the obtained porcine bristle meal, if compared for example with soybean meal, is still characterised by a very poor digestibility which considerably limits its use as ingredient for animal feed (Bertsch and Coello, 2005; Mazotto et al., 2017). With this in mind, the nutritive value of BPH_A and BPH_B was characterized as a means to assess the potential of pig bristle protein hydrolysates for use as fishmeal replacement in fish feed formulations. To this end the amino acid composition of porcine bristle meal and bristle protein hydrolysates was evaluated and the obtained results are illustrated in Table 1.

Table 1 Proximate analysis, in vitro digestibility, PDCAAS and BV of starting porcine bristle meal (PBM) and end products BPH_A and BPH_B

	PBM	BPH _A	BPH _B
Total Nitrogen [g/(100 g)]	14.62	15.18	15.19
Crude Fat [g/(100 g)]	7.1	4.2	4.1
Crude Ash [g/100 g]	1.9	0.9	0.7
Free AA [g/100 g]	Not det.	11.4	8.8
<i>In vitro</i> digestibility (%)	< 30	91.4	91.2
PDCAAS	0.12	0.34	0.35
BV	35.8	63.8	73.6

Both BPH_A and BPH_B were rich in glutamate, aspartate, glycine, leucine and proline whereas minor amounts of histidine and methionine were detected in each of the two protein hydrolysates. Proline is regarded as a conditionally essential AA in promoting fish growth and feed intake, especially in

juvenile and sub-adult fishes (Li et al., 2009). For instance, Zhang et al. (2006) observed that the amount of proline found in muscle of alevin rainbow trouts was dependent upon dietary proline and that endogenous synthesis was not sufficient to satisfy the proline requirement of this particular type of fish. It has also been suggested that dietary glutamate supplementation is likely to increase the antioxidant capacity of fish gut (Jos et al., 2009). For example, Zhao et al. (2015) conducted a feeding trial experiment on grass carps (*Ctenopharyngodon idella*) and demonstrated that the inclusion of glutamate improved growth performance and increased the intestinal antioxidant capacity, the digestive and absorptive ability of the species under investigation. Aspartate is an important energy substrate and an essential building block for purine nucleotide synthesis in all cell types of aquatic animals (Li et al., 2009). Recently, Gonzalez-Silvera et al. (2018) studied the impact of the dietary administration of feed supplemented with aspartate on the humoral systemic immune response of meagre (*Argyrosomus regius*) specimens when stressed by air exposure. Their results pointed out that feeding meagre with an aspartate-supplemented diet could prevent or at least reduce the decrease in seric peroxidase levels caused by air exposure. Regarding glycine, Shamushaki et al. (2007) demonstrated that this amino acid was linked to the stimulation of feed intake in Persian sturgeon juveniles. Additionally Takeuchi (2007) speculated that glycine could play a pivotal role in the osmoregulatory response of aquatic animals, in particular shellfishes, to environmental stress. Among others, leucine is considered one of the amino acids which are indispensable (i.e., essential) for a balanced fish diet, since it is known that suboptimal levels of dietary leucine can reduce growth performance and feed utilization of several aquatic species (Ren et al., 2015). During the course of some feeding trials, it was understood that leucine was capable to regulate the target of the rapamycin (TOR) signalling pathway, gluconeogenesis and lipogenesis in rainbow trout hepatocytes (Lansard et al., 2010; Lansard et al., 2011). Moreover, in a more recent study on grass carps, Deng et al. (2014) reported that optimal supplementation levels of leucine ameliorated both fish

growth performance and the antioxidant capacity of the feed. Interestingly, Trp, Tyr, and Ala which are all hydrophobic amino acids were the three most abundant found in the free amino acid fraction. Additionally, the other four hydrophobic amino acids of intermediate size (Phe, Met, Leu and Ile) were just following in terms of relative abundance within the free amino acid portion of the two obtained protein hydrolysates. In particular, if we look at their chemical structure, both Trp and Tyr contain large aromatic side chains, while Ala is characterized by the existence of a small aliphatic residue as its side group. Given these facts, the presence within the final hydrolysates of considerable amounts of both large as well as small hydrophobic amino acids seems to point out respectively the chymotrypsin-like specificity and elastase-like character of some of the proteases involved in the breakdown of the keratinous substrate. On the contrary, free amino acids with positively charged side chains were completely absent when considering Lys and His and present in a very little amount with respect to Arg which shows that proteolytic enzymes with trypsin-like specificity were most likely not present within the proteolytic cocktail (Perona and Craik, 1997; Demidyuk et al., 2017). In analogy to what was observed by Wang and Parsons (1997) in the case of steam-cooked hog hair meals, it could be stated that the hydrothermal pretreatment might have been partially responsible for the elevated content of leucine and valine found in both bristle protein hydrolysate. When considering the N-to-protein ratio for quantifying the crude protein content in biomass a value equal to 6.25 is frequently employed. Nevertheless, the arbitrary use of this estimate should be considered with caution since the calculation of the crude protein content from the concentration of the total nitrogen measured with the Kjeldahl method could easily result in an overestimation of the proteinaceous fraction present within the analysed sample. Having this in mind, in the present study we determined a new value for the N-to-protein ratio. To explain, it was assumed that the total amino acid content of the dried PBM was equivalent to its actual crude protein content. Therefore, based on the amino acid profile obtained for the PBM (see Table 2) it was calculated that the

amount of proteic matter present in the keratin-rich biowaste was about 75% of its dry weight. Thus, relying on this estimate we calculated an N-to-protein factor of 5.13. As a consequence, the crude protein content in the original PBM and in the two produced protein hydrolysate samples was obtained by multiplying the total nitrogen content of the PBM, BPH_A and BPH_B times 5.13. Hence, the crude protein content in the thermally treated hog hair powder available at the start of the experiments, BPH_A and BPH_B was 75.0, 77.8 and 77.9 % (w/w) respectively. Considering that both BPHs were prepared using only the culture supernatant, that is the post-hydrolysis solid residue was removed before freeze-drying the final products, it can be stated that the first and the second biodegradation strategies enabled to recover, respectively, 50.0 and 52.7% of the proteic material contained in the keratin-rich starting by-product. *In-vitro* pepsin digestibility was also measured. Each of the two obtained protein hydrolysates displayed a much higher digestibility value (i.e., 91.4% for BPH_A and 91.2% for BPH_B) compared to the original hair meal (< 30%). Data regarding amino acid profile and *in vitro* digestibility of both the original PBM and the two obtained protein hydrolysates BPH_A and BPH_B were employed to evaluate PDCAAS for each of the amino acids contained within the three nutrient meals. A feed that contains a source of dietary proteins characterized by a PDCAAS value of 1.0 is considered of high quality because it satisfies the requirements for essential amino acids of the farmed fish. From a nutritional point of view, the inclusion in the aquafeed of a protein mix that has a score higher than 1.0 brings no advantage, since the amino acids which are present in excess are not metabolized by the aquatic organism. In the first place, when considering the composition of the three products, it is evident that the lowest values for the PDCAAS were obtained in the case of the methionine which, indeed, was the main limiting amino acid present inside the prepared protein hydrolysates. Later on, histidine, lysine and cysteine could become the next limiting building blocks. Despite that, the two porcine protein hydrolysates exhibited PDCAAS values that were noticeably ameliorated with respect to those of the non-enzymatically

digested bristle meal. To explain these findings it should be considered that the *in vitro* digestibility of both biologically treated products was largely enhanced with respect to the starting material. In addition, the BVs of the produced BPH_A and BPH_B were respectively 1.8 and 2.1 times greater than that found for the initial thermally denatured bristle powder which clearly points out the superior nutritional quality of the two obtained protein digestates. For instance, both BPH_S exhibited BVs that were comparable to those determined in the case of hydrolysates obtained from the proteolysis of sodium caseinate and whey proteins (Sindayikengera and Xia, 2006), seed proteins (Pastor-Cavada et al., 2011) and fish proteins (Abdul-Hamid et al., 2002), just to name a few examples. Recently, it has also been suggested that protein hydrolysates obtained through chemical, enzymatic and microbiological degradation of keratinous waste materials, such as chicken feathers, could represent an alternative and inexpensive complex nitrogen source for the cultivation of microorganisms (Taskin and Kurbanoglu, 2011; Stiborova et al., 2016; Benesova et al., 2017). Indeed, in a typical biotechnological process for the production of fuels and chemicals the price of the nitrogen source can constitute a large fraction of the total manufacturing cost (Pleissner and Venus, 2016). Besides, undefined media normally include peptones, which form a complex source of organic nitrogen, beneficial for the cultivation of microorganisms (Pasupuleti and Demain, 2010). Therefore, protein hydrolysates, such as those obtained during the course of this experimental work through biodegradation of residual pig bristles, could be tested, within the context of a future investigation, to evaluate the potential for their application in replacing conventional peptones for microbial fermentation.

Table 2 Relative distribution of Amino Acids (AA) in protein hydrolysates expressed in mg AA per g Crude Proteins (CP)

Amino Acid (AA)	Total AA (mg/g)			Free AA (mg/g)		
	BPH _A	BPH _B	PBM	BPH _A	BPH _B	
Ala	45.6	42.1	46.1	21.7	15.9	
Arg	45.2	48.0	89.5	3.6	2.7	
Asp	67.1	68.2	63.5	6.3	5.0	
Cys	15.9	18.0	50.3	2.0	1.8	
Glu	133.7	136.1	146.7	28.2	23.2	
Gly	53.6	59.4	70.3	0.0	0.1	
His	7.8	8.1	13.1	0.0	0.0	
Ile	24.2	24.6	35.2	7.5	6.5	
Leu	54.1	54.2	75.7	19.7	16.7	
Lys	25.6	26.2	37.3	0.0	0.0	
Met	7.7	8.3	7.3	3.3	3.1	
Phe	25.6	22.1	31.9	9.0	4.4	
Pro	53.0	53.7	76.7	7.0	2.1	
Ser	43.6	48.6	87.3	0.0	0.0	
Thr	26.2	29.8	58.3	0.0	0.0	
Trp	7.5	7.7	9.7	3.8	3.6	
Tyr	36.6	32.1	36.3	17.7	12.0	
Val	45.8	46.2	60.1	16.6	14.2	
Total	719	733	1000	147	112	
Total essential	225	227	329	60	49	

Table 3 Protein digestibility-corrected amino acid scoring (PDCAAS) considering essential amino acids composition of bristle protein hydrolysates

Essential amino acid (EAA)	EAA profile (mg/g)			EAA reference (mg/g) *	Amino acid score (AAS)			PDCAAS		
	S1	S2	PBM		S1	S2	PBM	S1	S2	PBM
Arg	45.2	48.0	89.5	39.5	1.15	1.22	2.26	1.05	1.11	0.68
His	7.8	8.1	13.1	21.1	0.37	0.38	0.62	0.34	0.35	0.19
Ile	24.2	24.6	35.2	28.9	0.84	0.85	1.22	0.76	0.78	0.37
Leu	54.1	54.2	75.7	39.5	1.37	1.37	1.92	1.25	1.25	0.58
Lys	25.6	26.2	37.3	63.2	0.40	0.41	0.59	0.37	0.38	0.18
Met	7.7	8.3	7.3	18.4	0.42	0.45	0.40	0.38	0.41	0.12
Met + Cys	23.7	26.2	57.6	28.9	0.82	0.91	1.99	0.75	0.83	0.60
Phe	25.6	22.1	31.9	23.7	1.08	0.93	1.34	0.99	0.85	0.40
Phe +Tyr	62.2	54.2	68.1	47.4	1.31	1.14	1.44	1.20	1.04	0.43
Thr	26.2	29.8	58.3	28.9	0.91	1.03	2.02	0.83	0.94	0.60
Trp	7.5	7.7	9.7	7.9	0.95	0.98	1.23	0.87	0.89	0.37
Val	45.8	46.2	60.1	30.8	1.49	1.50	1.95	1.36	1.37	0.59

* NRC (2011) recommendation based on values obtained from studies conducted using test diets based on purified ingredients (Lall and Dumas, 2015).

4. Conclusions

In this work the full keratinolytic potential of the filamentous bacterium *Amycolatopsis keratiniphila* D2 was exploited to effectively direct the biological degradation of pretreated pig bristles to keratin protein hydrolysates. Indeed, an effective biorecovery process which integrates two stages in series was developed: in the first stage both proteolytic and keratinolytic enzymes were produced while in the second the previously obtained crude enzymes were employed for the biotransformation of the keratinic biowaste into a protein-rich keratin digestate. Moreover, it was revealed that keratinolytic hydrolysis of the porcine bristle meal significantly augmented its in vitro nutritional value making the produced bristle hydrolysates potential candidates in replacing, at least partially, more expensive protein sources in fish feed formulations. Overall, we believe that the bioconversion strategy described in this work truly constitutes a major attempt in paving the way for the sustainable reutilization of nutritionally valuable proteins which would remain otherwise untapped and irremediably trapped within the structure of this hair-type hard-to-degrade keratin biowaste.

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Chapter 5

Characterization of two novel proteases associated with the ability of *Amycolatopsis keratiniphila* to degrade keratinous slaughterhouse by-product

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1. Introduction

Keratinous by-product materials represent a large environmental challenge, but also hold a potentially enormous quantity of nutritional resources (Korniłowicz-Kowalska and Bohacz, 2011). Keratinous by-products are produced mainly from slaughterhouses, where slaughtering of for example poultry and pigs for meats, produce feathers, bristles and nails in great quantities (Mahro and Timm, 2007; Korniłowicz-Kowalska and Bohacz, 2011). At the molecular level many keratinous materials are built from proteins, some consisting of >90% protein, which should hold great nutritional value (Mahan and Shields. 1998; Mahro and Timm, 2007). Many keratinous materials, especially α -keratin materials, like hair, hooves, horns, bristles and nails, are however recalcitrant by nature and remain mostly undigested if used unprocessed as feed. This is in part due to the high levels of cross-links found in these materials (disulfide and isopeptide bonds), which conveys the resistance to proteolytic digestion by the protease found in the digestive tract of most animals (Marshall et al., 1991). There are however a number of microorganism (mainly fungi and bacteria), that are capable of degrading keratinous materials (Bockle et al., 1995; Korniłowicz-Kowalska and Bo-

hacz, 2011; Bohacz, 2017). These microorganisms utilize specialized proteases called keratinases, often in combination with systems for cleavage of the disulphide bonds (Böckle and Müller, 1997). Keratinases are not represented by a single family of protease, but within the S8 and M36 peptidase families, many members have been found to have keratinolytic activities (Brouta et al., 2002; Vermout et al., 2017). A bacterium named *Amycolatopsis keratiniphila* subsp. *keratiniphila* has been isolated by hair baiting and has subsequently been shown to degrade feathers and wool and grow on these as the sole carbon and nitrogen sources (Al-Mussallam et al., 2003). Little is known about the bacterium and what mechanisms it uses for keratin degradation, which is why we chose to study it in more detail and try to elucidate these mechanisms. In this study we grow *A. keratiniphila* on steam treated slaughterhouse by-product consisting of pig bristles and nails. From the culture supernatant two proteases were purified, characterized and were hypothesized to play a role in the degradation of the slaughterhouse by-product. The two proteases were both shown to belong to the S1 peptidase family, but showed widely different primary structure, temperature profiles and specificity. At least one of the proteases shows promise within biotechnological applications for keratin degradation, possibly as a combinatory treatment with other proteases.

2. Materials and methods

2.1.Slaughterhouse by-product

The slaughterhouse by-product used as carbon and nitrogen source in the growth cultures as well as for producing azokeratin for assaying was supplied by DAKA, Løsning, Denmark. The by-product mainly consists of pig bristles and nails, which have been chopped, steam treated (150 °C, 6 bar, 20 min), dried and crushed into smaller particles.

2.2.Microorganism and bacterial growth conditions

Amycolatopsis keratiniphila D2^T (strain number: DSM 44409) was obtained as freeze dried pellet from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. *Amy-*

colatopsis keratiniphila D2^T (D2) was regrown on LB media. Glycerol stocks were produced by growing D2 in LB medium overnight and mixing 500 µl culture with 500 µl 50% v/v sterile filtered glycerol. Stocks (1 ml) were stored in cryo-tubes at -80 °C.

Overnight cultures of *Amycolatopsis keratiniphila* were produced by inoculation of glycerol stocks into 10 ml LB in 50 ml sterile NuncTM tubes and incubated for 48 h at 30°C, while shaking at 180 rpm. From the overnight culture 1 ml was used to inoculate 100 ml keratin liquid medium, containing 10 g/L crushed pig bristle and nails, 5 mM NaCl, 5 mM NaH₂PO₄/Na₂HPO₄, 1 mM MgCl₂, 0.01mM CaCl₂ and 1µM ZnCl₂ at pH 7.5, in 500 ml shaking flasks. The cultures were incubated at 30°C, while shaking at 200 rpm. Cultures were generally grown for 4-5 days, while the culture was sampled periodically until keratinase activity had reached its peak.

2.3.Azokeratin preparation

Azokeratin was prepared by dyeing the same slaughterhouse by-product as used for growth of *Amycolatopsis keratiniphila*. Before dyeing the slaughterhouse by-product, it was milled in a coffee grinder to crush bigger pieces of pig nail, to include this in the azokeratin substrate. The milled keratin product (15 g) was dyed by suspension in 1 L distilled water, while 100 ml 1.19 M NaH₂CO₃ solution was added while stirring. Simultaneously, 8.65 g sulfanilic acid was dissolved in 0.12 M NaOH (200 ml), followed by the addition of 1.7 g NaNO₂, 10 ml 5.0 M HCl and 10 ml 5.0 M NaOH while stirring. After a few seconds of mixing, the solution was added to the suspension containing the milled slaughterhouse by-product and stirred for 10 min, before vacuum filtering using filter paper. The dyed keratin by-product (azokeratin) was washed twice in 1 L mQ water (first 30 min incubation, afterwards incubation overnight). The filtered azokeratin was freeze-dried to produce the finished azokeratin.

2.4. Azokeratin and keratin azure assay

To assay for activity on the slaughterhouse by-product, the azodyed by-product (azokeratin) was used. To each sample 12.5 mg azokeratin or 5 mg keratin Azure and 450 μ l 50 mM Tris-HCl, pH 8 (adjusted at incubation temperatures) was added to a 2 ml round bottom Eppendorf tube. Substrate and buffer were heated to the incubation temperature (37 °C and 55 °C), followed by the addition of 50 μ l sample to be assayed. To blank samples 50 μ l buffer was added. Samples were incubated for 1 h (unless otherwise indicated) while shaking at 850 rpm. After ending the incubation the samples were filtered through a 96-well filter plate (0.22 μ m) equipped with a vacuum manifold (Merck Millipore), filtering off residual insoluble substrate and stopping the reaction. The progress of the reaction was measured, by transferring of 200 μ l filtrate to a 96-well plate, followed by measuring absorbance on a plate reader (Powerwave XS; BioTek) at 415 nm (azokeratin) or 595 nm (keratin azure).

2.5. Assaying free thiols

A stock solution of 2 mM 5, 5'-dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent) was prepared in 50 mM tris-HCl, pH 7.5. For free thiol determination, 50 μ l sample was mixed with 50 μ l Ellman's reagent. The sample was then incubated for 2 min at 25 °C before absorbance was measured at 412 nm. A standard curve was created (0.01 mM–0.1 mM dithiothreitol (DTT)) with the purpose of determining free thiol concentrations in culture supernatant.

2.6. Protease purification

Cultures were grown for 4 days at which point peak activity of the culture supernatant towards azokeratin was observed, and insoluble species in the culture were harvested by centrifugation (10,000 g, 10 min). The culture supernatant was decanted and if necessary stored at -20°C. Proteins in the culture supernatant were precipitated by addition of solid $(\text{NH}_4)_2\text{SO}_4$ to reach 70% saturation on ice. The sample was centrifuged (10,000 g, 10 min) to harvest the precipitated material and the superna-

tant was discarded. The pelleted precipitate was dissolved in 25 mM tris-HCl, 1 mM CaCl₂, 1 mM MgCl₂ at pH 8 to a final volume corresponding to 5% of the original culture supernatant volume. The sample was centrifuged (15,000 g, 10 min) to remove any undissolved material. To remove any remaining (NH₄)₂SO₄, the sample was buffer exchanged using a 10 kDa spin filter (Amicon® Ultra 15 ml centrifugal filter, Merck Millipore), where the precipitate was transferred into the same buffer as used for dissolving the pellet. Once more the sample was centrifuged (15,000 g, 10 min) in preparation for cation exchange purification. The sample was applied to a cation exchange column (Resources S, 6 ml, GE Healthcare Life Sciences), with a 25 mM tris-HCl, 1 mM CaCl₂, 1 mM MgCl₂, pH 8 solution as running buffer (A solution) and 25 mM tris-HCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 M NaCl pH 8 solution as elution buffer (B solution). The sample was applied to the column with a flow of 6 ml/min and after about three column volumes, a gradient was setup, with an increase of 1% B solvent pr. min. Fractions (3 ml) were collected, and evaluated with regards to their keratinase activity using both the azokeratin assay and the keratin azure assay. Finally, fractions showing activity in the assays were pooled together. The pooled samples were concentrated and buffer exchanged on the 10 kDa spin filter into 50 mM sodium acetate/acetic acid, 1 mM CaCl₂, 1 mM MgCl₂, pH 4, after which the sample was centrifuged (15,000 g, 10 min) in preparation for the anion exchange purification. The sample was applied to an anion exchange column (Resources S, 6 ml, GE Healthcare Life Sciences), with a 50 mM sodium acetate/acetic acid, 1 mM CaCl₂, 1 mM MgCl₂, pH 4 solution as running buffer (A solution) and a 50 mM sodium acetate/acetic acid, 1 mM CaCl₂, 1 mM MgCl₂, 1 M NaCl, pH 4 solution as elution buffer (B solution). Fractions were assayed for activity on azokeratin as well as analysed on Nu-PAGE gel to estimate purity. Fractions containing each of the proteases were pooled and concentrated on a 10 kDa spin filter and stored in the 50 mM sodium acetate/acetic acid buffer, pH 4 (-20°C).

2.7. Identification of proteases by matrix-assisted laser desorption/ionization time of flight and LC-MS/MS

The purified proteases were run on a Nu-PAGE gel and the gel bands believed to contain the proteases, were excised using a scalpel and subjected to in-gel trypsin digestion. Gel pieces were washed in 40% ethanol to remove coomassie stain and then shrunk in 100% acetone, which was then completely removed by evaporation. To the gel piece, 50 μ l 10 mM DTT in 100 mM NH_4HCO_3 , pH 8 was added followed by incubation for 45 min at 56°C after which the solution was removed and free thiols were reacted with iodoacetamide, by addition of 50 μ l 55 mM iodoacetamide in 100 mM NH_4HCO_3 and incubated in the dark (25°C, 30 min). The supernatant was removed and gel pieces were again shrunk by addition of 100% Acetone. After removal of acetone, tubes were placed on ice and added 5 μ l 12.5 ng/ μ l trypsin (MS grade, Pierce™, Thermo Fischer Scientific) followed by incubation on ice (30 min). To each sample 20 μ l of a 20 mM NH_4HCO_3 solution was added and samples incubated at 25°C overnight. Each sample was then acidified by addition of 2 μ l 2% trifluoroacetic acid, samples were prepared for LC-MS/MS by desalting using homepacked stage-tips with C_{18} material (Empore™, 3M™, 3M company) and then vacuum-dried. Peptides were dissolved firstly in 2.5 μ l 2% formic acid followed by addition of 7.5 μ l ultrapure water.

Initial analysis of the digests were performed on using matrix-assisted laser desorption/ionization time of flight (MALDI-tof/tof Ultraflex II, Bruker Daltonics) to check the quality of the samples. Samples were then analyzed by LC-MS/MS and data were recorded in a data dependent manner, on an Orbitrap Fusion™ Tribrid™ (Thermo Scientific). An EASY nLC-1000 liquid chromatography system (Thermo Scientific) was coupled to the mass spectrometer through an EASY spray source, and peptide separation was performed on 15 cm EASY-spray columns (Thermo Scientific) with 2 μ m size C_{18} particles and inner diameter of 75 μ m. The mobile phase consisted of solvents A (0.1% formic acid) and B (80% acetonitrile in 0.1% formic acid). The initial concentration of solvent B

was 6%, and a gradient was applied to reach the following concentrations: 14% B after 18.5 min, 25% B after a further 19 min, 38% B after a further 11.5 min, 60% B after a further 10 min, 95% B in 3 min and 95% B was then maintained for another 7 min. The total length of the gradient was 70 min. The full scans were acquired in the Orbitrap with a resolution of 120,000 and a maximum injection time of 50 ms was applied. For the full scans, the range was set to 350-1500 m/z. From the full scans, parent ions were selected based on the top 10 most abundant ions and were sequentially sent for fragmentation with an isolation window of 1.6 m/z (Kelstrup et al., 2012), and were then added to an excluded list for 60 sec. For the MS/MS scans the resolution was set to 120,000 and a maximum injection time of 80 ms. The ions were fragmented in a higher-energy collision dissociation cell with normalized collision energy of 32% and analysed in the Orbitrap.

Data was analyzed using PEAKS 8.0 (Bioinformatics Solutions Inc.). The MS spectra were processed and analyzed using PEAKS workflow, with *de novo* sequencing of MS/MS spectra followed by database search against a sequence library. A sequence library was constructed from a trypsin digest pattern of the proteome of *Amycolatopsis keratiniphila* subsp. *keratiniphila*, allowing for unspecific cleavage at one of the peptide termini and a maximum of three missed tryptic cleavages within peptides. A build in contaminant database was simultaneously searched. Mass tolerance was set to 15 ppm for the parent ion and 0.02 Da for the fragment ions. Carboxymethylation of cysteine was set as a fixed modification, while deamidation of glutamine and asparagine, acetylation of protein N-terminus and oxidation of methionine were set as variable modifications. The false discovery rate on the peptide and protein level was not allowed to exceed 1%. Sample purity was based on a relative abundance estimate, calculating the ratio between the average intensity of the three most intense peaks for a given protein.

2.8. Bioinformatics analysis

Signal peptides for translocation of proteases were predicted using the SignalP (ver. 4.1) webserver (Petersen et al., 2011). Domain prediction was performed using the Conserved Domain Database online prediction webserver (Marchler-Bauer et al., 2017). Identification of similar protein sequences was performed using the BLAST online server at NCBI and alignment of the top 10 sequences was performed using the CLC Main Workbench 7 software. The sequence of human trypsin was obtained from Uniprot (UniProtKB - P07477 (TRY1_HUMAN)).

2.9. LC-MS/MS analysis of protease specificity

Peptides used for protease specificity LC-MS/MS analysis, were prepared by digestion of the DAKA slaughterhouse by-product, from which the peptides were purified. Samples were prepared by addition of 5 mg DAKA product and 450 µl tris-HCl, 1 mM CaCl₂ to a 2 ml round bottom tube, followed by heating to 37 °C and addition of 50 µl 0.4 µM protease solution. Samples were incubated for 2 h, at which point the samples were centrifuged (5 min, 15,000 g, 4°C). The resulting supernatants were then transferred to new tubes and 2% trifluoroacetic acid was added to a final concentration of 0.2% trifluoroacetic acid. The protein concentration in the digests was estimated based on absorbance at 280 nm ($E_{280\text{ nm}} = 1\text{ L}\cdot\text{g}^{-1}\cdot\text{cm}^{-1}$) and a volume containing 10 µg of peptides was used for peptide purification using the stage tip procedure described above. Samples were analyzed by LC-MS/MS as describe above. Spectra were analyzed using PEAKS 8.0 (Bioinformatics Solutions Inc.) essentially as describe above, with the exceptions being that the sequence library was made from *Sus scrofa* genome and no cleavage specificity was set, for the *in silico* digest. Peptides matched to confidently identified proteins, were used for specificity analysis. Redundant peptides identified as chemically modified versions were only counted once, while peptides identified in more than one protein where counted once per identified protein. For C-terminal peptide analysis, the C-terminal amino acid of the peptide was taken as a representative of the P1 specificity of the

protease. For N-terminal peptide analysis, knowledge about the position of a peptide match and knowing that a cleavage must have occurred at the N-terminal of the peptide as well, allowed for prediction of P1 site specificity based on this knowledge.

2.10. Temperature and pH activity profile

Synthetic substrates were used for investigation of the activity at varying temperature and pH for both proteases. For the C-like protease the synthetic substrate N-succinyl-Ala-Ala-Val *p*-nitroanilide (Sigma Aldrich) was used and for the T-like protease the substrate N_α-Benzoyl-L-Arg *p*-nitroanilide (Sigma Aldrich) was used. Assays for determination of the temperature profiles of both proteases were performed in 50 mM NaH₂PO₄/Na₂HPO₄ at pH 7.5 with 2 mM substrate and the protein concentrations of 0.9 μM (C-like) or 0.4 μM (T-like). Before addition of the proteases, buffer and substrate mixture (96 μl) was preheated to the desired temperature (20 – 70 °C). After addition of protease (4 μl) samples were incubated while shaking (500 rpm) for 10 min (T-like) or 15 min (C-like). The reaction was stopped by addition of 100 μl 500 mM acetic acid pH 4. The pH profile assays were conducted essentially as describe above, with the only changes being the buffers used at the varying pH and the incubation temperature being 37°C for both proteases. Buffers were acetic acid (pH 4), maleic acid (pH 5–6), Na₂HPO₄/NaH₂PO₄ (pH 7), tris-HCl (pH 8–7) and NH₄H₂CO₃ (pH 10–11). All buffers were adjusted to a temperature of 37 °C. Absorbance was measured (Powerwave XS; BioTek) at 405 nm by transferring 200 μl sample to a 96-well plate.

2.11. Chemical additives

A series of chemical additives were tested to see how they affect the protease activity towards the synthetic substrate. The metal ions tested were Mg²⁺, Ca²⁺, Zn²⁺, Cu²⁺, Mn²⁺ and Fe²⁺ (1 mM and 10 mM). Inhibitors tested were EDTA and PMSF (1 mM and 10 mM). In the case of metal ions and inhibitors, the proteases were incubated together with these on ice for 30 min before addition of substrate. The protease activity was also tested in the presence of detergents, SDS (0.1 and 1.0 v/v

%), Triton X-100 (0.5 and 2.5 v/v %) and Tween 20 (0.5 and 2.5 v/v %). For the detergents there was no pre-incubation. The reaction time was 10 min (T-like) or 15 min (C-like) and reaction temperatures were 37°C (T-like) and 60°C (C-like). The reaction was stopped by addition of 500 mM acetic acid pH 4 (1:1 v/v). Absorbance was measured (Powerwave XS; BioTek) at 405 nm by transferring 200 µl sample to a 96-well plate.

2.12. Active site titration

The concentrations of active sites in protease stocks were determined using N-trans-cinnamoylimidazole (Proteinase K, >30 Units/mg, Molecular biology grade, Sigma Aldrich®, Merck) and Subtilisin A, Sigma chemical company), *p*-nitrophenyl trimethylacetate (C-like) and 4-Methylumbelliferyl *p*-guanidinobenzoate (trypsin and T-like). Solid N-trans-cinnamoylimidazole (Alfa Aesar, Thermo Fischer Scientific) was dissolved in acetonitrile to make a stock solution (10 mM). Proteinase K (Molecular biology grade, Sigma Aldrich®, Merck) and Subtilisin A were dissolved in 25 mM acetic acid pH 4, to a concentration near 300 µM based on weight. Active sites were determined for proteinase K and subtilisin A by mixing 2 µl N-trans-cinnamoylimidazole stock solution with 100 µl 25 mM sodium acetate/acetic acid pH 5 in a cuvette (1 cm pathlength) and the spontaneous hydrolysis was recorded (335 nm), until a stable linear hydrolysis was recorded after which 10 µl of protease stock was added. The absorbance decrease was recorded for 2 min after addition of protease to ensure that linear extrapolation was possible. The number of active sites was determined based on the cleavage of N-trans-cinnamoylimidazole using a molar extinction coefficient at 335 nm ($9.37 \cdot 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$) (Schonbaum et al., 1961). The active site concentration was generally 50–65% of that determined by absorbance measurements at 280 nm. Solid *p*-nitrophenyl trimethylacetate (Sigma Aldrich®, Merck) was dissolved in acetonitrile to make a stock solution (10 mM). A stock of C-like protease ($\approx 65 \text{ µM}$) was prepared and the concentration was estimated based on absorbance at 280 nm. Active sites were determined by mixing 2 µl *p*-

nitrophenyl trimethylacetate stock solution with 100 μl 50 mM tris-buffer pH 9 in a cuvette (1 cm pathlength), and the spontaneous hydrolysis was recorded (405 nm), until a stable linear hydrolysis was recorded after which 20 μl of protease stock was added. The absorbance increase was recorded for 2 min after addition of protease to ensure that linear extrapolation was possible. The number of active sites was determined based on release of *p*-nitrophenol using a molar extinction coefficient for the phenolate at 405 nm ($18.3 \cdot 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$) (Biggs, 1954). The active site concentration was generally 85-90% of that determined by absorbance measurements at 280 nm. Solid 4-Methylumbelliferyl *p*-guanidinobenzoate (Sigma Aldrich) was dissolved in acetonitrile to make stock solution (100 μM). A stock solution of T-like protease ($\approx 8 \mu\text{M}$) and porcine trypsin ($\approx 9 \mu\text{M}$, Sigma Aldrich) was prepared based on absorbance at 280 nm. Active sites were determined by mixing 2 μl 4-Methylumbelliferyl *p*-guanidinobenzoate stock solution with 198 μl 50 mM tris-buffer pH 8 in a fluorimeter cuvette and the spontaneous hydrolysis was recorded by excitation at 365 nm (slit width 2.5 nm) and emission observed at 445 nm (slit width 10 nm), until a stable linear hydrolysis was recorded after which 20 μl of protease stock was added. The emission increase was recorded for 2 min after addition of protease to ensure that linear extrapolation was possible. The number of active sites was determined based on a standard curve of free 4-methylumbelliferone (0.01–0.1 μM). Triplicates of all the active site titrations were performed, giving two times the standard error of the mean lower than 5% for all measurements.

2.13. Protease activity towards different substrates

Stocks of the five proteases (T-like protease, C-like protease, Trypsin, Proteinase K and Subtilisin A) were prepared, based on the active site titrations, to a concentration of 0.4 μM active sites. Five substrates were used for activity analysis (Azokeratin, keratin azure, azocasein, duck feathers and dog fur) Duck feathers and dog fur were defatted in 96% Ethanol by soaking the material for 5 min after which it was dried at 30 °C for 24 h. Azokeratin and keartin azure assays were performed as

described above and carried out at both 37 °C and 60 °C. Azocasein assays were performed by preparing a 10 mg/ml azocasein solution (1.35 ml) in 50 mM tris-HCl, 1 mM CaCl₂ pH 8 (pH adjusted at incubation temperatures), 150 µl protease stock was added and samples incubated at 37 °C. The reaction was sampled periodically (100 µl), these samples were added 10 µl TCA 100% w/v and then the samples were centrifuged (10 min, 15.000 g). The supernatants (75 µl) were transferred to 96-well plates, added 125µl 0.5 M NaOH and absorbance was measured at 415 nm. The duck feather and dog fur were tested essentially as described for the keratin azure. Degradation was evaluated by measurement of absorbance (280 nm) on sample supernatant indicating release of protein.

3. Results and discussion

3.1.Purification and identification

Amycolatopsis keratiniphila has previously been found to grow on keratinous materials like feather-meal and wool (Al-Mussallam et al., 2003), which is the reasoning behind investigating its ability to grow on steam treated slaughterhouse by-product consisting mainly of pig bristles and nails. Furthermore, culture supernatant containing proteolytic enzymes produced by *A. keratiniphila* has shown great capability towards degradation of keratinous by-products, prompting the investigation of protease expressed by *A. keratiniphila*. The results show that *A. keratiniphila* grows well on the keratin media, exhibiting about a two day lag phase before keratinase activity is observed and free protein levels increase (Figure 1). It is interesting to note that during the first two days of incubation, the free protein present in the media is not initially consumed by the organism before keratinase activity is observed. This is in good agreement with previous studies, indicating that the presence of what is considered easier available nitrogen and carbon sources, does not suppress the keratinase and protease secretion by *A. keratiniphila* (Al-Musallam AA et al., 2003). During the course of two to three days (48 – 110 h growth period) the keratinase activity and free protein in-

crease to maximum level after which the keratinase activity decreases, while the level of free protein stays constant to the end of the experiment (160 h).

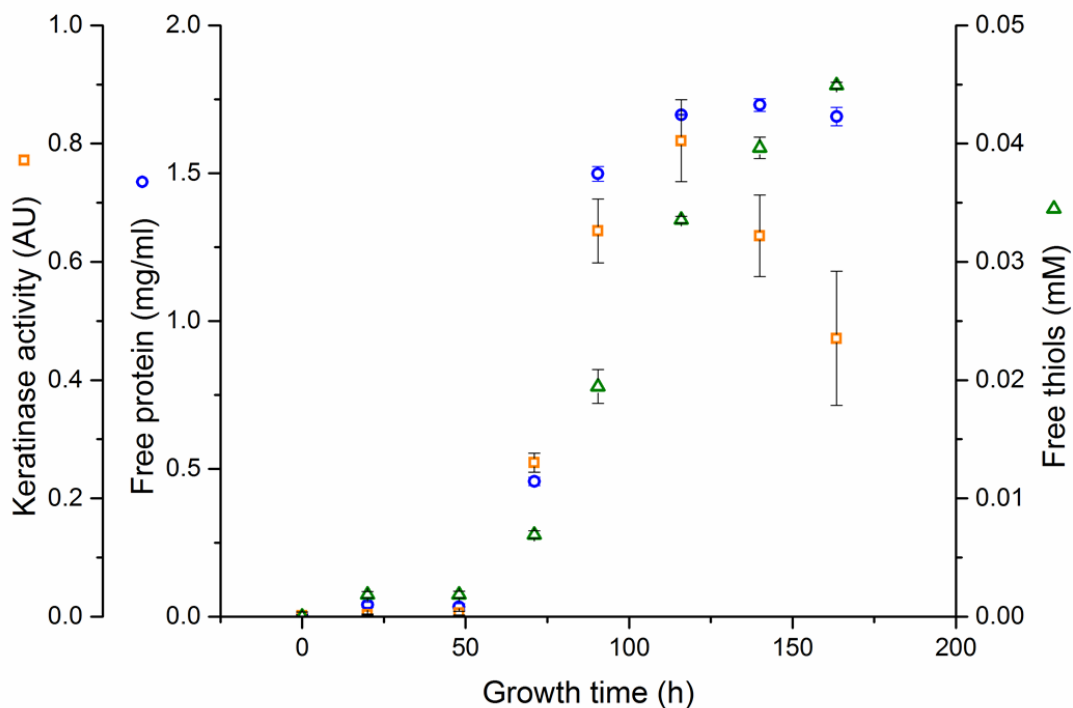


Figure 1: Growth of *Amycolatopsis keratiniphila* on keratinaceous slaughterhouse waste. *Amycolatopsis keratiniphila* was grown on slaughterhouse waste and three parameters were followed throughout the growth period. Activity towards azokeratin (**circle**), the concentration of free protein (**square**) and the concentration of free thiols (**triangle**) all showed a lag phase until 75 h, after which the value of all three parameters increased. The azokeratin activity peaks at 120 h after which it decreases, while the free protein concentration stays at level and the free thiol concentration increases steadily. The free protein concentration has been subtracted the background concentration present in the medium at 0 h. The error bars correspond to the standard deviation of three replicates.

Whether there is a metabolic reason behind this decrease in keratinase activity or a reduction of the bacteria viability is the cause of this observed decrease, is not known. Furthermore, it is noteworthy

that the keratinase activity peak co-aligns with levelling off of the free-protein concentration in the supernatant, indicating that at the present conditions the keratinases in the culture are not able to release more protein from the keratin substrate even though the activity levels are high. At the end of the measurement period (160 h) the free protein concentration in the culture supernatant reaches 1.69 mg/ml, whereas the protein background (protein at 0 h) amounts to 2.64 mg/ml protein. This means that 36.6% of the slaughterhouse by-product has been converted to free protein, assuming that 72.6% of the by-product constitutes protein, which is based on amino acid analysis (DAKA, 2017. PM90 Pig Bristle Meal). Another common mode of degradation used by bacteria and fungi on keratin rich materials is reduction of the high number of disulphide bonds found in the matrix and between the individual keratin molecules (Böckle and Müller, 1997). We measured the concentration of free thiols in the supernatant, and although levels of free thiols increase simultaneously with the increase of the measured activity towards azokeratin and the free protein concentration, it does not follow the exact same profile. If we correlate the amount of free thiols measured with the amount of free protein present at the end of the growth experiment (160 h) and the amino acid profile of the by-product, the free thiols detected here only account for about 4% the theoretical amount expected if the free protein released during degradation has the same amino acid profile as the substrate. This indicates that reduction of disulphide bridges might not be an approach utilized by *A. keratiniphila* for decomposition of the keratin material. A different reason could be that the free thiols are still present in the insoluble substrate and not detected in the soluble fraction or that the bacterium utilizes another method of disulphide bond breakage (sulphitolysis).

In an attempt to dissect the protease profile expressed by *A. keratiniphila* during growth on the slaughterhouse by-product, proteases were purified from a culture supernatant harvested at peak keratinase activity (\approx 120 h). A zymogram of the supernatant shows three major and at least two minor proteolytic species active under these conditions (Figure 2).

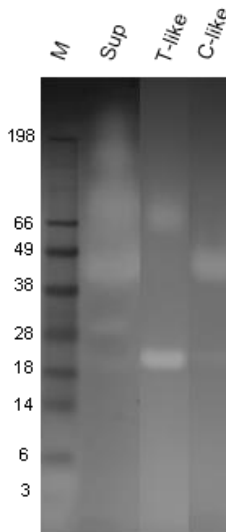


Figure 2: Casein zymogram showing the protease profile of *Amycolatopsis keratiniphila* culture supernatant and the two purified proteases. The culture supernatant shows the clear presence of proteases capable of cleaving casein resulting in bright clearing zones on the gel. The two purified proteases also show clearing zones corresponding to some of the major clearing zones in the supernatant sample. Due to the running conditions the clearing zones are not necessarily representative of the molecular weight of the proteases. Furthermore the T-like sample contains two clearing zones, which is hypothesized to be due to a folded and an unfolded fraction of the protease.

An ammonium sulphate precipitation served to concentrate the supernatant and resulted in good recovery of the activity as judged by activity towards both the azokeratin and keratin azure (Table 1). The concentrated supernatant was fractionated by applying it to a Resource S column (cation exchange) at pH 8. As can be seen in Figure S1, about 75% of the activity (azokeratin and keratin azure) is recovered in the flow-through column together with most of the other proteins and impurities (280 nm). It also observed that later fractions (B15–B7), representing proteins separated from the majority of the other species in the culture supernatant, contain proteins active towards the keratin substrates. In this study we have chosen to focus on the proteins separated from the flow-through in this study. Fractions containing azokeratin activity (B15–B7) were pooled and subjected to yet another separation on the Resource S column, but at pH 4, resulting in better separation of the active species (Figure S2). The majority of the activity is defined in two peaks with maxima around fractions A15 and B7, which by SDS-PAGE resulted in two clear bands of similar size, fraction A15 containing a smaller protein (≈ 26 kDa) than the one in the B7 fraction (≈ 28 kDa) (Figure 3).

Table 1: Table summarizing the purification process, followed by the activity of samples towards azokeratin and keratin azure. The purification of the two proteases was followed by the activity measurement towards both azokeratin and keratin azure, showing the same activity yield at each step of the purification. Absolute activity is the absorbance measured during the assay multiplied by the amount of sample for a given purification step.

<i>Purification step</i>	<i>Azokeratin</i>		<i>Keratin azure</i>	
	Absolut activity	Yield (%)	Absolut activity	Yield (%)
Culture supernatant	1691	100	478	100
AMS precipitation	1727	102	495	104
Sample for IEC (pH 8)	1600	94.6	489	102
Sample for IEC (pH 4)	402	23.8	135	28.3
T-like protease	133	7.9	-	-
C-like protease	177	10.5	-	-

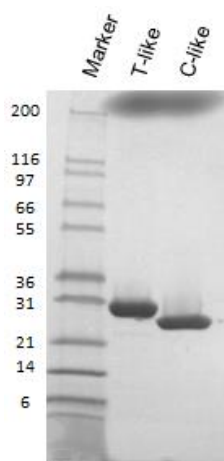


Figure 3: Nu-PAGE gel showing the purified proteases. The two purified proteases show almost equal size with the T-like protease being slightly larger as judged by these results. The amount of both proteases loaded on the gel is 10 µg and they are thus quite pure as visualized here.

The protein in the 28 kDa band seems to possess at least one disulphide bridge since the migration of the protein is altered by the presence of a reducing agent, in the sample buffer (Figure S3). The migration length of the 26 kDa protein band was not affected, which is in agreement with the presence of only a single possible disulphide bridge within the protein structure (Pos85 and Pos101 of

the alignment, Figure S8). Zymograms of the two purified proteases indicate that these can be two of the major proteolytic species in the supernatant (Figure 2 and Figure S4). The proteins were identified by trypsin digestion, followed by LC-MS/MS analysis, identifying both proteases as having a trypsin like sequence (Conserved Domain Database search engine (Marchler-Bauer et al., 2017), data not shown) and thus belonging to the S1 family of proteases (Figure S5 and 6). The protease in the 28 kDa band was termed T-like protease and showed above 60% sequence identity with three trypsin-like proteases in the Uniprot/Swiss-prot databases (Figure S7). The protease in the 26 kDa band was termed C-like protease and only showed low identity (< 35%) with proteins from the Uniprot/Swiss-prot databases (Figure S8). Proteases belonging to the S1 family are rarely associated with activity on keratinous substrates (Mitsuiki et al., 2004), thus this discovery is unusual and broadens the spectra of proteases that could be considered important for keratin degradation as they are active towards both the azokeratin and keratin azure. The LC-MS/MS analysis furthermore indicates that the purified protease samples contain less than 2% impurities (data not shown).

3.2.Characterization

First the proteases were used for digestion of the slaughterhouse by-product material, from which the proteolytic peptides released into the supernatant were subjected to LC-MS/MS analysis, revealing the specificity of the proteases at the P1 site. For the T-like protease the specificity was clearly characteristic of a trypsin-like protease, with Arg and Lys being heavily represented at the C-termini (P1) of the identified peptides (Figure S9). Analysing the primary structure of the T-like protease with the Conserved Domain Database and aligning it with other trypsin-like proteases (Figure S7), reveals that the substrate coordinating amino acid is Asp, similar to all trypsin-like proteases used for the alignment. The fact that an Asp is present at the exact proposed site of substrate coordination, as is seen for representative trypsin proteases, gives further credibility to the trypsin-like specificity observed. As for the C-like protease specificity, the same analysis did not yield as

clear a picture, though it clearly differed from the T-like protease (Figure S10). Most notably is the apparent specificity towards Ala, Val and Ile, which is distinctly different from that of the T-like protease. Furthermore, we also see an apparent specificity towards Arg, however this apparent specificity might be caused by impurities in the substrate and the instrument's bias towards more positively charged ions. Similar results have been observed in other studies utilizing LC-MS/MS for protease specificity determination (Gupta et al., 2010). Analysing the primary sequence of the C-like protease reveals that it shares relatively low sequence identity ($\leq 35\%$) with proteins from the Uniprot/Swiss-prot database (Figure S8). It is also observed that while the protease aligns with trypsin-like proteases it does not share the Asp as the coordinating residue, but instead has a Ser. This reinforces the MS data, which show different specificity compared to the T-like protease. Furthermore, when the concentration of active sites in solutions containing the C-like protease was measured it did not show activity towards the fluorometric chymotrypsin substrate 4-methylumbelliferyl *p*-trimethylammoniocinnamate, which simulates a large hydrophobic amino acid side chain (data not shown). On the other hand it did show activity towards the substrate *p*-nitrophenyl trimethylacetate, that is a synthetic substrate developed to simulate an elastase substrate, with a smaller hydrophobic side chain. From the mass spectrometry data we can also verify that both proteases cleave different keratins in the slaughterhouse by-product, among them the krt81, krt84, krt85, krt31, krt33b and krt34, which all have been characterized as “hard keratins”, located in different parts of hair and nails (Bragulla and Homberger, 2009) (data not shown). The specificity of the two proteases was further verified by cleavage of the synthetic substrates N-succinyl-Ala-Ala-Val *p*-nitroanilide (C-like) and N $_{\alpha}$ -Benzoyl-L-Arg *p*-nitroanilide (T-like), which were also used for further characterization of the proteases.

The temperature activity profiles of the two proteases further discriminate them from each other, showing the widely different optima (Figure 4A and 4C).

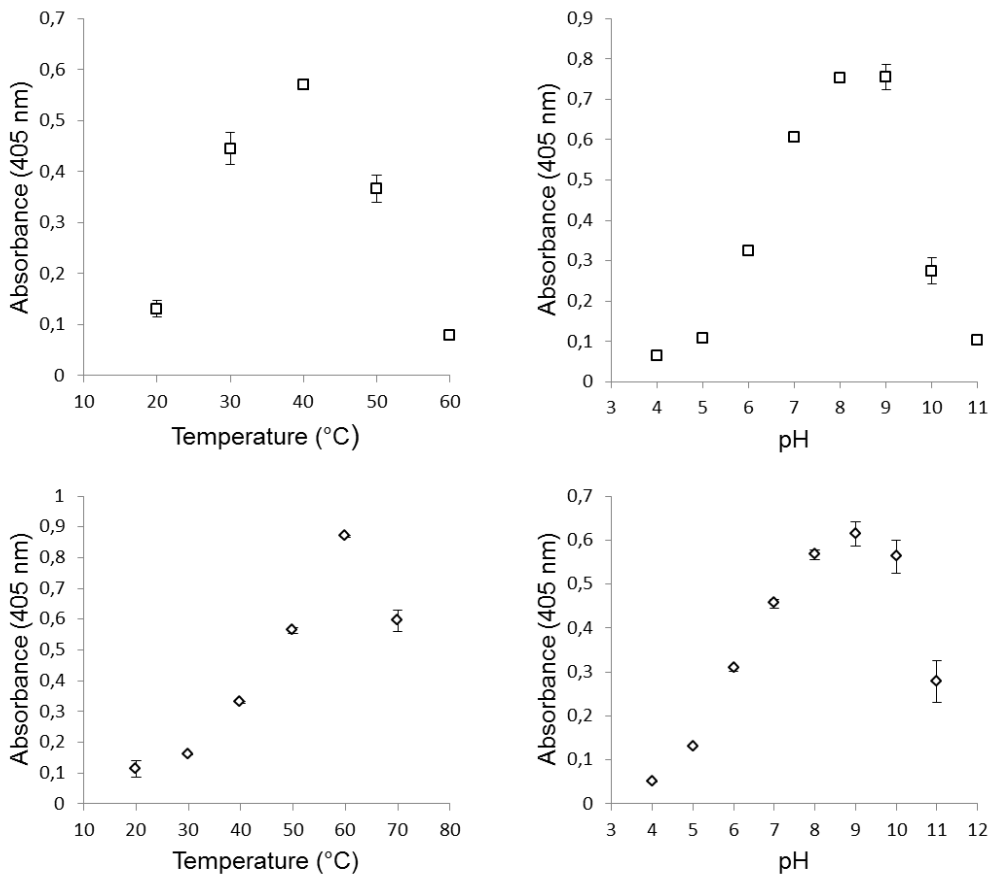


Figure 4: Temperature and pH profile of the T-like and C-like proteases. The temperature profile of the two proteases are widely different with the T-like (A) protease having a maximum around 40 °C and the C-like (C) around 60 °C. The pH profiles of the two proteases are more similar with both the T-like (B) and the C-like protease (D) having maxima in the range between pH 8–9. The error bars are the standard deviation of three replicates.

The T-like protease has an optimum around 40 °C, which is lower than the trypsin counterparts found in mammals (Buck et al., 1962a; Buck et al., 1962b). The C-like protease optimum is around 60 °C. Compared to the T-like protease, the optimum temperature of the C-like protease is much further from the optimal growth temperature of *A. keratiniphila*, which is around 28 °C (Al-Mussallam et al., 2003). It has however been shown that the protease activity of the culture superna-

tant from *A. keratiniphila* grown on slaughterhouse by-product, as the one used in this study, has a temperature optimum around 60 °C (Chapter 3). This indicates that the C-like protease could play a role, when culture supernatant is used for hydrolysis of slaughterhouse by-product at elevated temperatures (Chapter 3). The dependency of protease activity on pH gave similar results for both proteases (Figure 4B and 4D), with optimum around neutral to slightly alkaline pH (pH 8–9). The protease activities were then tested in the presence of different metal ions and chemical additives (Table 2). Both proteases showed a slight increase in activity upon addition of divalent ions Mg^{+2} and Ca^{+2} , while the Cu^{+2} and Zn^{+2} has a distinctly negative effect on the activity. This could indicate that the two proteases bind Mg^{+2} and more likely Ca^{+2} , as observed for other proteases in the S1 family (Sipos and Merkel, 1970). The dependence on metal ions is further established for the T-like protease, as 10 mM EDTA also affects the activity negatively. Surprisingly, 10 mM EDTA has no effect on the C-like protease's activity. As expected, both proteases are inhibited by the serine protease inhibitor PMSF. Additionally the disulphide bond breaking reagents DTT and sulphite have negative effects on the activity of both proteases. This indicates that both proteases contain disulphide bonds that are important for the activity, likely due to their importance in maintaining the structural integrity. The dose shift from 1 to 10 mM of the reagents does however not seem to have much effect on the activity, indicating that 10 mM of these reagents can be used in combination with the proteases for degradation purposes. While the non-ionic surfactants have a positive effect on the activity of the proteases at low concentrations, this effect is lost at higher concentration and results in a slightly negative effect. SDS has a clear negative effect on the proteases in the concentration range tested.

Additive/Concentration	C-like		T-like	
	1mM	10mM	1mM	10mM
Tris	100 +/-2.5	100 +/-2.8	100	100 +/-4.1
Ca ⁺²	100.1 +/-1.3	106.5 +/-1.5	105.1 +/-2.9	117.6 +/-0.8
Mg ⁺²	93.1 +/-2.4	104.5 +/-0.1	100.8 +/-9.0	105.5 +/-2.7
Zn ⁺²	93.0 +/-1.5	78 +/-0.9	50.1 +/-1.4	24.8 +/-1.4
Fe ⁺²	95.7 +/- 3.5	ND	102.9 +/-2.1	ND
Cu ⁺²	99.4 +/-1.6	72.9 +/-1.0	101.5 +/-2.8	63.1 +/-1.3
Mn ⁺²	98.2 +/-2.6	97.5 +/- 3.2	103.0 +/-1.1	104 +/-3.6
EDTA	96.1 +/-4.8	100.5 +/-1.9	97.5 +/-2.7	53.3 +/-10.5
DTT	77.2 +/-5.4	80.2 +/-2.5	97.4 +/-3.2	90.5 +/-1.5
SO ₃ ⁻²	92.8 +/-6.9	87.9 +/-1.8	90.9 +/-3.5	91.5 +/-2.3
PMSF	65.1 +/-1.0	39.8 +/-1.7	94.2 +/-2.3	55.0 +/-2.4
	0.5% v/v	2.5% v/v	0.5% v/v	2.5% v/v
Triton X-100	126.3 +/-3.1	95.5 +/-4.2	110.3 +/- 3.9	90.0 +/-2.4
Tween 80	123.9 +/-1.7	94.5 +/-2.1	117.5 +/- 4.0	92.0 +/-0.8
	0.1% v/v	0.5% v/v	0.1% v/v	0.5% v/v
SDS	20.4 +/-6.3	0.8 +/-0.9	30.8 +/-2.4	0.7 +/-0.3

Table 2: Activity of the two proteases in the presence of different metal ions and chemical additives.

The activity of the two proteases at the different conditions is represented as percent activity relative to a reference sample (50 mM Tris-HCl, pH 8). The errors are the standard deviation of three replicates and ND means no data is available.

The ability of the two proteases to degrade three different proteinous materials was tested and compared to trypsin, proteinase K and subtilisin from *Bacillus licheniformis* near the temperature opti-

imum of the T-like and the C-like proteases. All protease stocks were analysed for active site content, to ensure equal concentrations of active sites in the reactions. Proteinase K showed the highest activity of all the tested proteases on azokeratin, both at 37 °C and at 55 °C (Figures 5A and B), which is to be expected considering proteinase K is known for its activity towards keratinous substrates (Ebeling et al, 1974). Neither the T-like nor the C-like protease outperformed trypsin over the duration of the experiment and C-like protease was the least active at 37 °C. At 55 °C the C-like protease is performing much better than at 37 °C, which is to be expected from its temperature profile, however it is not outperforming trypsin and is inferior compared to proteinase K. The activity assay using the more homogenous keratin substrate, keratin azure, showed some interesting tendencies (Figures 5C and 5D). Unlike the results with the azokeratin substrate, the C-like protease is being significantly more active, compared to the other proteases at 37 °C. While the T-like protease seems to be the least effective in degrading keratin azure, the subtilisin protease did not show any significant degradation compared to the background without any added protease, even after 40 h of incubation. The keratin azure substrate seems to favour the C-like protease, compared to the T-like protease, indicating that the two proteases have different specificities towards the two substrates. Proteinase K is again the most active of the proteases at 55 °C, but the C-like protease shows an interesting trend within the first 15 h by keeping a steady level of degradation which is only increasing at the 40 h point. This same tendency is also observed at 37 °C. Compared to proteinase K and trypsin, C-like protease has lower degradation within the first 7 h at 55 °C, however, at 15 h when the trypsin degradation efficiency decreases, the C-like protease's degradation is more or less unchanged throughout the experiment. Furthermore, as the proteinase K activity also begins to level off, the gap in absolute degradation between proteinase K and the C-like proteases is decreasing. In this setup the C-like protease thus seems to be close to being on par with proteinase K as a keratin degrader.

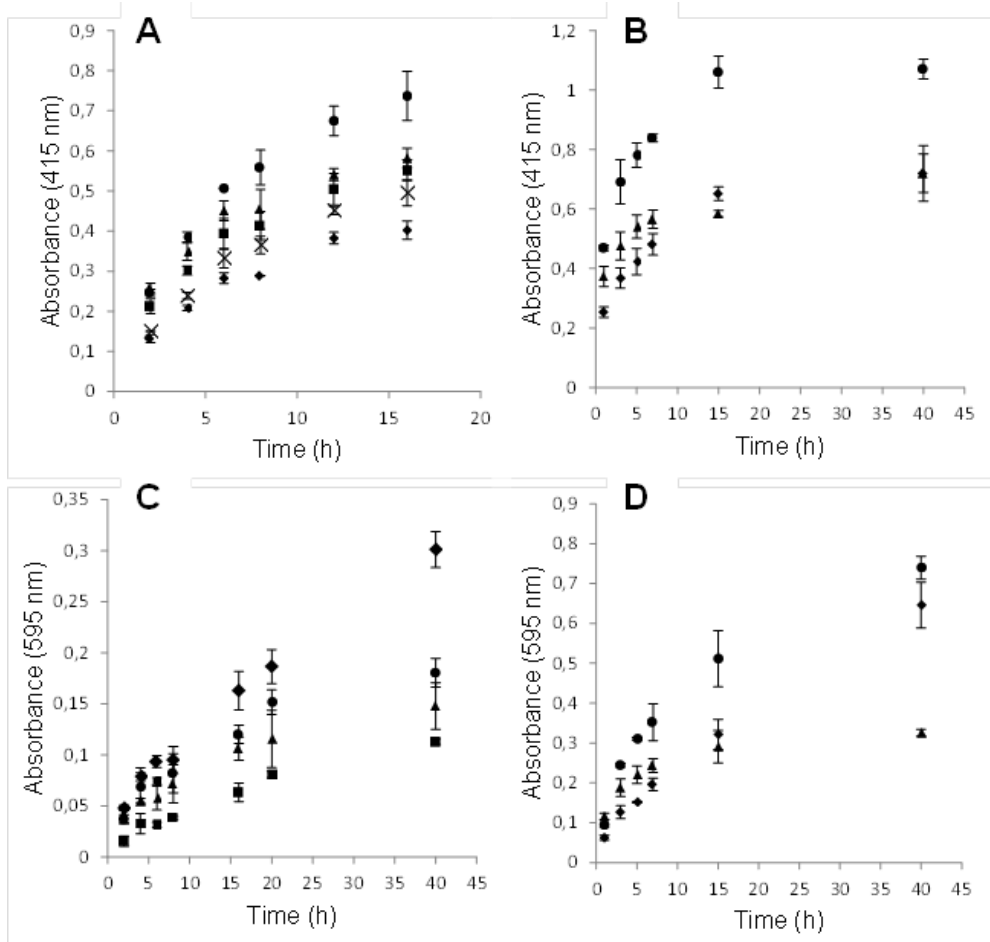


Figure 5: Degradation profile of five proteases with azokeratin and keratin azure as substrates. Azokeratin (A and B) and keratin azure (C and D) were degraded using the T-like protease (square), C-like protease (diamond), porcine trypsin (triangle), subtilisin A (cross) and proteinase K (circle) at two temperatures (37 °C and 55 °C). Proteinase K is shown to be the most effective at degrading azokeratin at 37 °C (A) and at 55 °C (B). However, of the two purified proteases the T-like one is the most effective of the two at 37 °C, while the C-like one is overall more effective at 55 °C (T-like not tested at 55 °C). Notably, it is observed that the C-like protease seems to be the most effective degrader of keratin azure at 37 °C, while the T-like protease is the least effective of the proteases that showed activity towards keratin azure (C). The subtilisin protease did not show any significant degradation of the keratin azure substrate. At 55 °C the C-like protease performs better than trypsin and at 40 h, it is almost as efficient as proteinase K (D). The error bars are the standard deviation of three replicates.

Assays on the universal protein substrate azocasein indicate that the two proteases in this study have the lowest specific activity towards azocasein, under the given conditions (Figure 6).

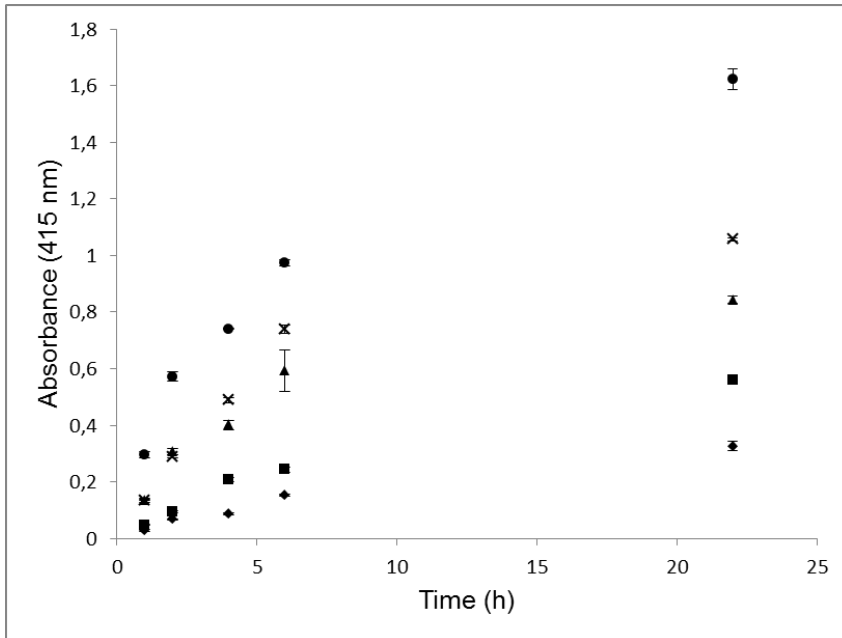


Figure 6: Degradation profile of five proteases with azocasein as substrates. The degradation of azocasein was done using the T-like protease (square), C-like protease (spades), porcine trypsin (triangle), subtilisin A (cross) and proteinase K (circle) at 37 °C. The two proteases purified in this study are clearly the slowest degraders of the universal protease substrate azocasein. The error bars are the standard deviation of three replicates.

This indicates that the T-like and C-like proteases have a higher specificity for the keratinous substrates than proteinase K, subtilisin and trypsin, if the ratio between keratin activity and casein activity is taken as a measure of this specificity for keratinous substrates (Gupta et al., 2012). On a molecular level these proteases would thus be interesting to further investigate, to obtain more knowledge on what interactions are responsible for conveying this apparent specificity towards the keratinous substrates. Testing the activity towards dog fur and duck feathers resulted in only proteinase K showing activity, and for the duck feathers activity was only recorded when incubated at

55 °C (data not shown). Thus, under the tested conditions the two proteases do not seem to show any noteworthy degradation of these keratin materials.

4. Conclusion

Two proteases were purified from the culture supernatant of *A. keratiniphila* grown on steam treated slaughterhouse by-product consisting mainly of pig bristles and nails. The proteases were purified based on their activity towards the azo-dyed slaughterhouse by-product and keratin azure. Both proteases were identified by LC-MS/MS and shown to belong to the S1 family of proteases. The proteases showed different physico-chemical properties and cleavage specificities. The T-like protease clearly showed close relations to trypsin-like proteases, as indicated by sequence alignment and it possessed a clear specificity towards Arg and Lys at position P1. The C-like protease only showed low sequence identity by alignment with proteins in the Swiss- and Uni-prot databases and had an apparent activity towards small to medium sized hydrophobic amino acids (Ala, Val and Leu). Both proteases showed significantly lower activity towards azocasein, compared to the other tested proteases, while being more on par when activity was measured towards the keratinous substrates azokeratin (slaughterhouse by-product) and keratin azure. These results points towards the purified proteases having an apparent higher specificity towards keratin substrates compared to the other tested proteases. It was furthermore verified by LC-MS/MS that the two proteases hydrolyze the keratin proteins in the slaughterhouse by-product, as peptides from these substrates were identified. It is unclear what biological role these two proteases play in the degradation process, but the C-like protease has potential as a keratin degrader, possibly in combination with other proteases at elevated temperatures (55 °C).

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Supporting Figures:

S1:

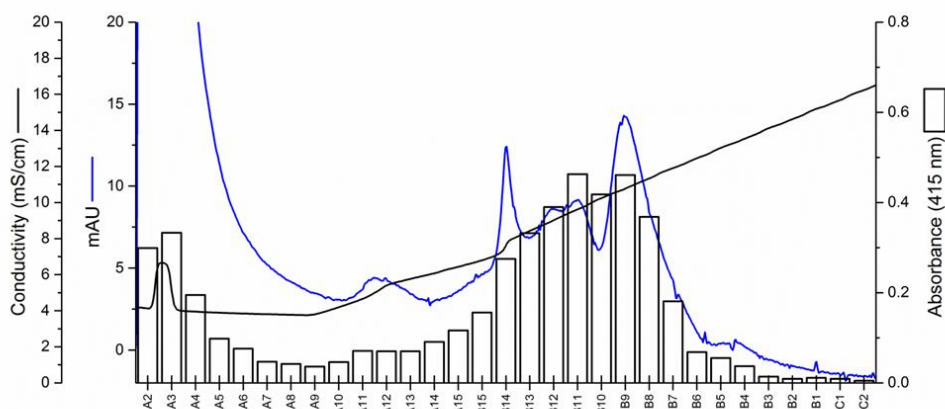


Figure S1: Cation exchange chromatography of proteins from culture supernatant, performed at pH 8. Culture supernatant was subjected to fractionation using a Resource S column, eluting proteins with a gradient of increasing NaCl concentration. Fractions were assayed for azokeratin activity and active fractions were pooled (fractions within the bracket, B15–B7). Fractions A2 and A3 were diluted 10 times with buffer before being used in the assay, thus absorbances should be multiplied by 10.

S2:

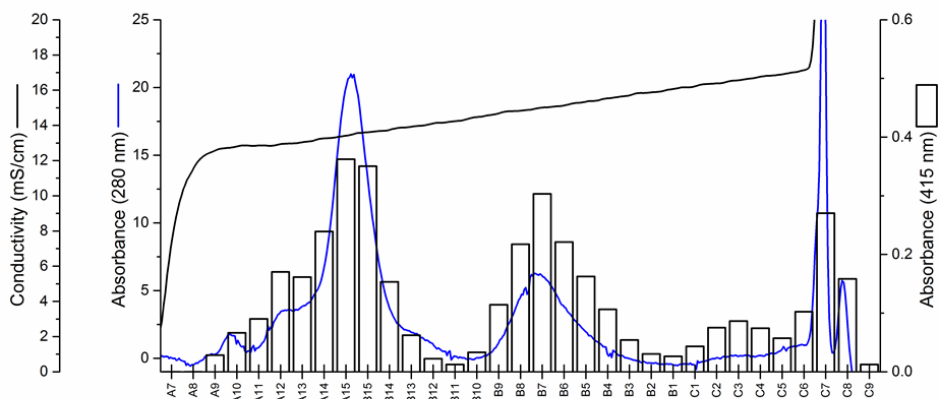


Figure S2: Cation exchange chromatography of proteins from culture supernatant, performed at pH 4. Pooled fractions from previous purification step were subjected to fractionation again using a Resource S column, but at pH 4, eluting proteins with a gradient of increasing NaCl concentration. Fractions were assayed for azokeratin activity.

S3:

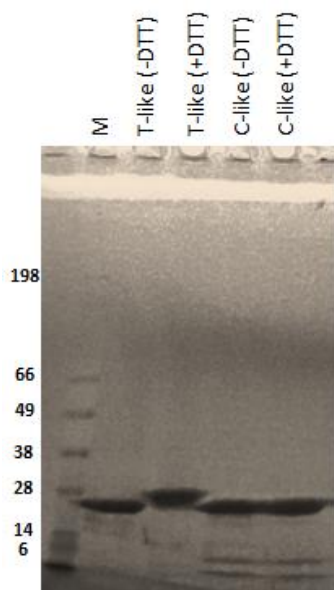


Figure S3: Nu-PAGE gel of the two purified proteases with or without reducing agents. Samples of the two proteases were run of a Nu-PAGE gel at reducing (+ DTT) and non-reducing (- DTT) conditions. The results indicate that the T-like protease contains disulfide bridges in the structure, while the C-like protease could contain disulfide bridges that alter the structure of the protease to a lesser degree compared to the T-like protease.

S4:

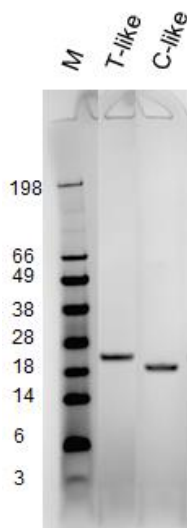


Figure S4: Nu-PAGE gel of the two purified proteases, with the gel being run at similar conditions as for the casein zymograms. Samples in this gel correspond to the same samples used in the casein zymogram of figure 2 and the gel is run similarly to the zymogram gel. The difference compared to figure 2 is thus only how the gel is run (lower voltage and low temperature). The proteins in these samples have been TCA treated, unlike for the zymogram and are thus assumed to be unfolded during the gel electrophoresis. It can be observed that the band in the T-like lane is of similar size as to one of the clearing zones observed on figure 2.

S5:



Figure S5: Primary structure of the T-like protease. Analysis of the primary structure of the T-like protease using the conserved domain database search engine and the Signal P online webserver indicates residues carrying important functions. A signal peptide was predicted to be cleaved off between Ala33–Glu34. Further processing was predicted to take place at Asp39–Val40. Following Val40 a trypsin-like domain was predicted with a substrate coordinating residue predicted at Asp209, responsible for the coordination of a Lys or Arg residue at the P1 site.

S6:

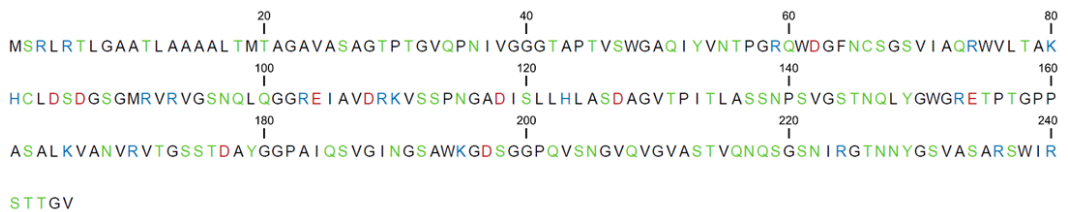


Figure S6: Primary structure of the C-like protease. Analysis of the primary structure of the C-like protease using the conserved domain database search engine and the Signal P online webserver indicates residues carrying important functions. A signal peptide was predicted to be cleaved off between Ala25–Ser26. Further processing was predicted to take place at Asn36–Ile37. Following Ile37 a trypsin-like domain was predicted with a substrate coordinating residue predicted at Ser192, responsible for the coordination of the P1 residue, which indicates that the specificity of the protease is not the typical trypsin specificity (Lys and Arg).

S7:

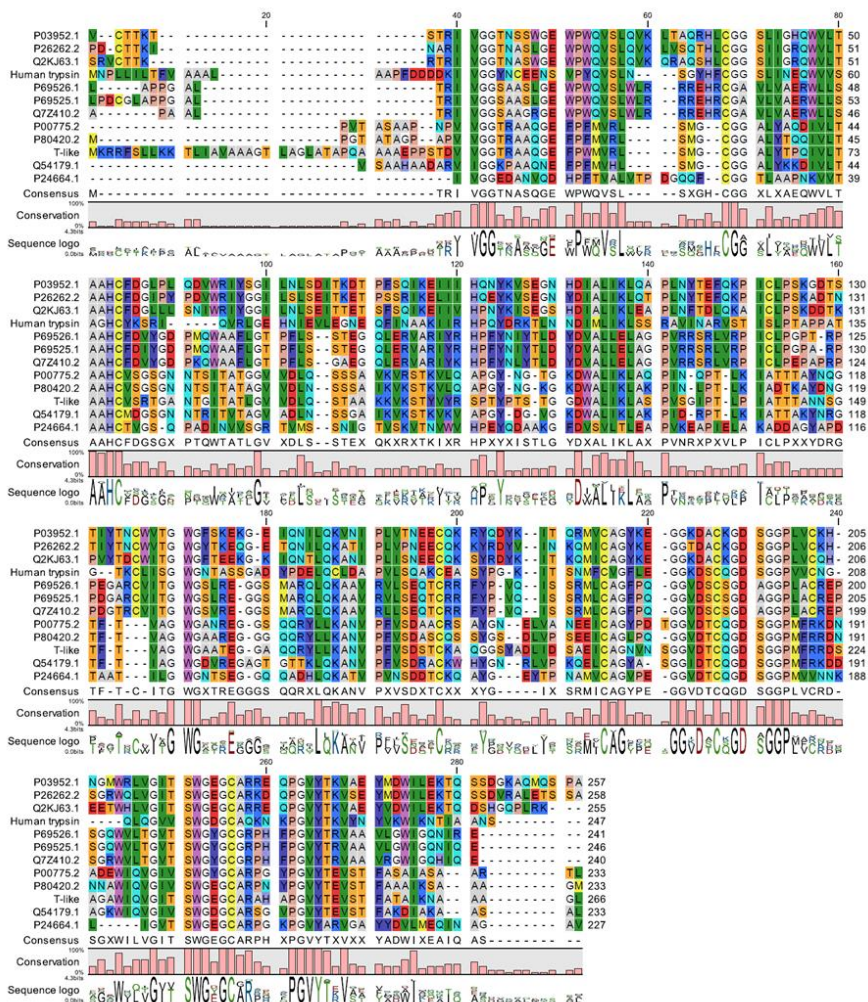


Figure S7: Alignment of the T-like protease with the top 10 hits in the BLAST of the protease against the Uniprot/Swiss-prot databases and human trypsin. The similarities between the proteins begin at position 40, where the presence of four hydrophobic amino acids is aligned in all proteins, resembling the N-terminal of a mature trypsin protease. At position 225 of the alignment the amino acid predicted to be responsible for the P1 specificity is found to be an Asp, which is similar to that of human trypsin and fits the results from the specificity analysis. Some of the blast hits have been truncated at the N-terminal to include only the trypsin domain, to shorten the alignment. Sequences are represented by their Uniprot accession number.

Figure S8: Alignment of the C-like protease with the top 10 hits in the BLAST of the protease against the Uniprot/Swiss-prot databases and human trypsin. The similarity between the proteins begins at position 54 and presence of four hydrophobic amino acids again aligns in all proteins, resembling the N-terminal of the T-like protease. At position 261 of the alignment the amino acid predicted to be responsible for the P1 specificity is found to be a Ser for the C-like protease, while for many of the other proteases it is Asp as for human trypsin. This indicates that the C-like protease might not have specificity for Arg and Lys. Notably the C-like protease also contains a deletion just prior to the coordinating residue (Pos252–Pos259). Sequences are represented by their Uniprot accession number.

Chapter 6

Project outcome and future perspectives

The economic model on which we have built the recent development and wellbeing that we enjoy in our modern society is to a large extent based on the unprecedented and totally uncontrolled exploitation of the non-renewable resources of our planet. In recent years, the constant rise in global carbon emissions, related global warming and consequent onset of climate changes on an ever more global scale has significantly contributed to reconsidering our relation with the planet earth. The quest for a more sustainable use of our limited “common home” resources has recently prompted a more serious effort towards the replacement of oil based feedstocks with renewable biomasses. Nevertheless, the primary focus of the biotech community has been in the direction of developing cost-effective microbial and/or enzymatic conversion technologies which could transform renewable plant-based agricultural residues into biofuels, biochemical products and biobased materials. Until now, very little attention has been paid to the development of methods and techniques which can support the effective recovery, upgrade and therefore reutilization of animal processing by-products. As a matter of fact, if we aim at successfully implementing the principles of the circular bioeconomy it will be fundamental to include also these other types of biomasses in the research effort. The transformation of keratinous waste streams into valuable products can be of particular interest when considering that a sustainable supply of protein is also needed in order to meet the growing protein demand especially with respect to protein-rich ingredients for animal feed.

The present PhD thesis has focused on the investigation of the microbial degradation of a hair-type hard-to-degrade keratinous material, namely porcine bristles, and the development of a biotechnological process that could effectively convert porcine bristles into protein-rich keratin hydrolysates.

Amycolatopsis keratiniphila D2, a potent keratin-degrading strain, was chosen as the model microorganism to investigate the degradation of thermally pretreated pig bristles.

In the first part of this work (Chapter 2) efforts were directed towards the systematic optimization of the microbial decomposition of pretreated porcine bristles employing the bacterial strain *A. keratiniphila* D2, with the objective of maximizing the levels of crude soluble proteins extracted during the course of the biodegradation process. In this case the production of keratinases and the hydrolysis of the keratinous waste material were carried out simultaneously in a single stage process. This required an environment suitable for stable growth of the microorganism, implying that the strain should experience conditions which support the synthesis of sufficient levels of extracellular keratinases. At the same time, the catalytic rate of the keratinolytic cocktail of secreted enzymes was maximized as a way to increase the productivity of crude soluble proteins during the bioconversion process. One of the major drawbacks when using this single stage approach is that one has to compromise between the possibility of accumulating larger amounts of keratin-specific proteases within the culture supernatant on the one hand, and the prospect of obtaining a higher rate of keratinous biowaste hydrolysis on the other hand, meaning that both phenomena could only take place in a system which was subjected to suboptimal conditions. Nevertheless, the above-mentioned challenge with the single stage approach was tackled by means of the newly developed approach described in Chapter 3.

The integrated strategy detailed in the second part of this work consisted of a two-stage microbial degradation process through which, in a single unit operation, both production of keratinolytic enzymes and hydrolysis of the keratinaceous substrate were conducted under optimal conditions. The developed method permitted to design and successfully run a cultivation process in which the growth of the bacterial strain, i.e. the stage controlling the extracellular accumulation of the keratin-specific proteases into the culture broth, was completely disengaged from the phase in which the

pretreated pig bristles were converted into soluble proteins, small peptides and free amino acids. Since the two phases involved in the biological degradation process were fully decoupled in time, both stages could be optimized independently. The economically feasible large-scale implementation of the enzymatic hydrolysis of keratinous biomass would require operating the process at high solids loadings. However, the handling of a large concentration of water insoluble solids (WIS), especially due to the low availability of free water, involves the formation of highly viscous slurries which poses a serious mixing challenge and severely limits the mass and heat transfer efficiency of the system. In order to maintain the concentration of non-hydrolysed keratinous particles within the stirred tank at a manageable level a fed-batch enzymatic hydrolysis process was tested. To this end, the solid substrate was split into three aliquots (40:40:20) and fed stepwise to the extraction vessel (reaching a cumulative solid loading of 15% w/v). Under the established conditions, at the end of the process, a highly-concentrated protein hydrolysate was obtained ($89.3 \text{ g}\cdot\text{L}^{-1}$ crude soluble proteins + $8.7 \text{ g}\cdot\text{L}^{-1}$ free amino acids). Additionally, it was demonstrated that the largest fractions identified inside the final bristle protein hydrolysate were those composed of small peptides and free amino acids.

In the following part of the thesis (Chapter 4), the potential use of a cell-free crude keratinases extract obtained from *A. keratiniphila* D2 was explored as another way of directing the biodegradation of the pretreated pig bristle powder. The enzymatic hydrolysis process was again operated in a fed-batch mode. Moreover, at this point in time the solid substrate was divided into four fractions of equal size (25:25:25:25) which were fed to the stirred tank every 24 hours to reach a total water insoluble solids (WIS) loading of 15% (w/w). The amino acid profile and *in vitro* digestibility test for the obtained bristle protein hydrolysates were utilized to calculate some *in silico* parameters which were in turn employed in performing a preliminary assessment of the potential of the enzymatically produced protein-rich digestates for use as fishmeal replacement in a fish feed formula-

tion. The experimental study revealed that the addition to the hydrolysis vessel of the whole quantity of enzyme entirely from the start, when compared to a stepwise enzyme addition, resulted in the best performance both in terms of extraction of crude soluble proteins ($73.2 \text{ g}\cdot\text{L}^{-1}$ vs $69.4 \text{ g}\cdot\text{L}^{-1}$) and with regard to the biological value (BV) of the obtained bristle protein hydrolysate powder (73.6 vs 63.8). It was also demonstrated that the recovered protein hydrolysates presented significantly augmented nutritional characteristics in comparison to the original porcine bristle meal, thereby highlighting the potentially analogous nutritional value of such keratinous protein digestates with respect to hydrolysates obtained from the proteolysis of sodium caseinate, whey, seed, and fish proteins, and last but not least soybean proteins. Hence, the enzymatic route to obtain hydrolysed keratin meals shows a potential for substituting a considerable fraction of the fish meal used in aquaculture feed formulations and, more in general, for becoming a protein supplement in animal feed.

In Chapter 5 the purification was described of two keratin-specific proteases isolated from the culture supernatant of *A. keratiniphila* D2 when grown on pretreated pig bristles and nails powder. Both keratinolytic enzymes were shown to belong to the S1 family of proteases despite exhibiting different physicochemical properties and cleavage site specificities. In addition, one of the two keratinases (the T-like protease) was closely related to trypsin-like proteases showing a clear preference towards Arg/Lys side chains at the P1 position. Besides, the C-like protease could not be distinctly identified by sequence alignment with proteins in the Swiss- and Uni-prot databases. Both purified enzymes demonstrated high specificity towards keratinic substrates when compared with other standard proteases. Finally, it was also pointed out that both keratin-specific proteases displayed keratinolytic capability with respect to the porcine bristle and nail meal.

To conclude, in the remaining part of this chapter few suggestions will be listed regarding what are considered some of the promising future directions that could be taken for this research to further rationally develop some of the necessary tools which will be needed to bring about the creation of a

successful waste biorefinery based on the sustainable transformation of keratin-rich residual biomass into biofuels, biochemical products and bio-based materials.

First of all, the major bottleneck that needs to be addressed in order to achieve the efficient and cost-effective bioprocessing of keratinous waste materials is that, at the current state of the art, microbial keratinase productivities and titres which are obtained with wild type strains in submerged fermentation systems are still very low. Thus, the production of some of the most powerful keratinolytic enzymes using molecular cloning and heterologous expression systems, hand in hand with the selection of a recombinant host fit for the purpose of producing large amounts of keratinolytic enzymes, will be of fundamental importance in paving the way to commercialization of the next generation of keratin-specific proteases.

Moreover, as emphasized previously, when operating the enzymatic hydrolysis at high solids loadings the resulting highly viscous slurry will definitely constitute a challenge in obtaining effective mixing and efficient heat transfer inside the bioreactor. Consequently, conventional stirred tank reactors with typical impeller configurations are not practical for this application because of the high stirring speeds that are needed to mix the slurry and keep the solids suspended. Gravitational or free-fall horizontal mixing systems such as scraped surface slurry bioreactors have proven to offer several advantages over typical stirred tank reactors. The horizontal scraping action of the impellers in a free fall principle reactor requires very low rotation rates and, hence, much less power while preventing particle settling and local accumulation of reaction products, as well as ensuring better enzyme distribution. Moreover, the scraping action of the blades maintains a clear reactor surface and, thereby, improves heat transfer. Within this context, in collaboration with Professor Navid Mostoufi from the University of Tehran, we are currently developing a computational fluid dynamic model to be used for gaining deeper insight into the mixing behavior of the keratinous particulate system within a horizontal scraped surface slurry reactor. The computational multiphase

flow model that is under development will then be employed, in a following stage of the investigation, as the starting point for the construction of a laboratory unit.

Additionally, it is important to consider that, at least in principle, it might not be possible to achieve complete hydrolysis of the keratinous substrate, not to forget also that one should take into account that very little is known about whether keratinolytic proteases could be inhibited by high concentrations of some of the products released during the breakdown of the keratinous biomass. Therefore, anaerobic digestion of the non-hydrolysed solid residue could represent an interesting course of action to further valorise the non-solubilised fraction obtained following the extraction of the crude proteins which were part of the keratinous waste macromolecular structure.

Finally, once they have been obtained, protein hydrolysates could be easily converted, as was demonstrated in this PhD thesis, into protein meal to be included in animal feed formulations, or further transformed into novel bio-based products such as biodegradable foaming agents (Bhavsar et al., 2017), regenerated keratin fibers (Xu and Yang, 2014), and keratin-based bioplastics (Jin et al., 2011; Dou et al., 2016), just to name a few.

APPENDIX A

Enzymatic Hydrolysis of Keratin: Process Understanding and Mechanistic Insights

1. Introduction

The objectives of the work described in this section were (i) to investigate the kinetics of enzymatic hydrolysis of keratin-rich waste material by monitoring the enzymatic stability at different temperatures; (ii) to explore the main behavior of the enzymatic hydrolysis at different solids loadings; (iii) to develop a mathematical model to describe the enzymatic hydrolysis of keratin.

Therefore, enzymatic hydrolysis was performed with a keratinolytic enzymatic cocktail which was produced by the filamentous bacterium *Amycolatopsis keratiniphila* D2, first reported by Al Mussallam et al. (2003). The mathematical model was based on Michaelis-Menten kinetic and reaction rate being affected by enzymatic deactivation and non-competitive inhibition. Different solids loadings were tested to model the decrease in reaction rate with increasing substrate conversion and model performance is tested by uncertainty and sensitivity analysis.

2. Materials and methods

2.1. Experimental methods

All hydrolysis experiments were carried out at pH 8.0 in a 50 mM Tris (hydroxymethyl)aminomethane) buffer. Thermally pretreated (150 °C, 6 bars, 20 min) pig bristles from DAKA SARIA group A/S (Løsning, Denmark) were used as the keratin-rich substrate. 10% trichloroacetic acid (TCA) was used to stop enzymatic reactions completely.

2.2. Enzyme preparation

Lab scale hydrolysis experiments were performed using a keratinolytic enzymatic cocktail obtained with the filamentous bacterium *Amycolatopsis keratiniphila* D2 (DSM 44409) (Al-Mussallam et al., 2003). *A. keratiniphila* D2 was initially cultivated in a mineral keratin medium having the following

composition: 0.75 g/L NaCl, 1.75 g/L K_2HPO_4 , 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.055 g/L $CaCl_2$, 0.010 g/L $FeSO_4 \cdot 7H_2O$, 0.005 g/L $ZnSO_4 \cdot 7H_2O$ and 1% w/w porcine bristle meal (PBM) powder at 28 °C and pH 7.2. After 4 days keratinases concentration reached the maximum value, therefore the culture broth was centrifuged at 4000 rpm for 40 minutes (Heraeus™ Multifuge™ X3R, Thermo Fisher Scientific) to remove cells and pre-filtered with a 10-12 µm paper filter (Sartorius™ Grade 288 Qualitative Filter Papers Disc) to remove larger residual particles and cellular debris. The final filtration was performed with a syringe filter of 0.45 µm (Sartorius™ Minisart® NML) to remove smaller non-solubilized particles. As a result, a cell-free crude keratinases extract was obtained at pH 8 and stored at -80 °C to retain enzymatic activity.

2.3. Cell-free crude keratinase extract thermal stability test

After reequilibrating at ambient temperature, the crude enzymatic cocktail was filtered again with a syringe filter (0.45 µm). 4 ml glass vials were filled with 2 ml of crude filtered enzymatic cocktail solution properly diluted in Tris buffer and were then closed with a plastic screw cap. Duplicate samples were treated in a thermoshaker (700 rpm) operated at 30, 40 and 50 °C, respectively. After thermal treatment samples were removed from thermoshaker at 2, 4, 6, 8, 10, 24, 32, 48, 60 and 72 hours. Samples were quenched in ice and immediately frozen at -80 °C for keratinolytic activity test at later stage.

Keratinase activity was assayed with azokeratin as a substrate by the following method. 200 µl of the thermally treated crude enzyme cocktail was diluted with the addition of 800 µl, 50 mM Tris buffer. The reaction mixture contained 200 µl of the adequately diluted enzymatic cocktail preparation in addition to 1400 µl of 50 mM Tris buffer, pH 8.0, with a final concentration of 10 g/l of azokeratin. The mixture was incubated for 15 minutes at 50 °C; samples were then quenched in ice and the reaction was stopped by the addition of 1600 µl TCA to a final concentration of 5 g/l azokeratin. After centrifugation at 12000 x g for 5 minutes, the absorbance of the supernatant was determined

at 440 nm. One unit of enzymatic activity was defined as the amount of enzyme that caused a change of absorbance of 0.01 at 440 nm when incubated for 15 minutes at 50 °C.

2.4. Hydrolysis experiments

The small-scale hydrolysis experiments were conducted in 4 ml glass vials. Substrate was weighed to in each vial (3%, 5% or 7% w/w), then 2 ml crude enzymatic cocktail preparation was added to each vial, and finally the vials were placed in a thermoshaker (40 °C, 600 rpm). Due to small volumes, no samples were drawn from the vials during the course of experiment, however three identical replicates for each data point were prepared to monitor enzymatic hydrolysis at different reaction times. At fixed time intervals, the corresponding vials were removed from thermoshaker and quenched in ice 200 µl supernatant was taken from each vial as sample to perform the PierceTM BCA protein assay to measure the protein concentration. The remaining vial contents were vacuum filtered with 55 mm WhatmanTM glass microfibre filters, dried overnight in the oven at 105 °C and weighed to determine residual substrate mass.

Hydrolysis experiments with a 1-hour reaction time were used to calculate the initial substrate consumption rate. These experiments were performed in triplicates and the initial substrate concentrations used in these experiments are shown in Fig. 2a. Batch hydrolysis experiments with a longer reaction time (72 hours) were carried out with 3, 5 and 7 w/w % solids loadings. The sampling times in the batch experiments were 2, 4, 8, 24, 48 and 72 hours. The experiments for longer reaction time were also carried out in triplicates.

In addition to the batch hydrolysis experiments, an enzyme adsorption test was also performed to determine the effect of the unproductive adsorption of the enzymatic cocktail at the very beginning of the reaction. Hydrolysis experiment with a 1-hour reaction time was used to calculate adsorbed portion of the enzymatic cocktail.

2.5. Kinetic model for enzymatic hydrolysis

Based on the experimental results, a model for the enzymatic hydrolysis of keratin in a batch process was proposed, and parameter estimation was performed to determine unknown parameters. To be able to construct the model, the following assumptions were made: (i) The substrate composition is structurally uniform and there is no distinction between the reactivity of different parts of the substrate; (ii) The non-keratinous material used in the experiments was assumed to be inert and there is no interaction between the enzyme and non-keratinous material that can affect the hydrolysis; (iii) Due to small volumes, the reaction mixture was perfectly mixed in the vials (iv) Finally, the adsorbed part of the enzyme at the very beginning of the hydrolysis was assumed to be inactive for the rest of the reaction time. In this study hydrolysis kinetics was described using Michaelis-Menten expression as a suitable model for biochemical reactions involving a single substrate.

In this study, the rate of reaction for the enzymatic system was expressed as the product of two independent differential equations. One equation was dependent upon the substrate concentration and described the reaction kinetics, whereas the other expressed the kinetics of enzymatic deactivation. Additionally, an inhibition factor was included to the first differential equation to capture the decrease in reaction rate with increasing reaction time. As a necessity to use Michaelis-Menten kinetics, it is assumed that substrate-enzyme complex maintains quasi steady state. It was also observed that the first hour of the hydrolysis is dominated by the enzyme adsorption and first-order reaction of the enzyme-substrate complex. Thus, parameters of Michaelis-Menten expression are estimated with the experimental results obtained in the first hour of hydrolysis to avoid reaction rate decreasing factors related to the substrate and enzyme behavior. Parameter estimation was performed with constant adsorption and kinetic parameters and kinetic model was developed in Matlab 2016 by using Particle Swarm Optimization Toolbox.

First of all, the mass balances for the substrate and soluble proteins are described by the following equations:

$$\frac{dC_K}{dt} = -r_K \quad (1)$$

$$\frac{dC_P}{dt} = f \cdot r_K \quad (2)$$

In equations 1 and 2, C_K is the keratin concentration (g/l), C_P is the protein concentration (g/l), r_K is the substrate consumption rate (g/l/h) and f is hydrolysis yield ($\text{g}_{\text{protein}}/\text{g}_{\text{substrate}}$), relating protein production to substrate consumption, with the remainder of the consumed substrate mass consisting of released fats, lipids, peptides etc. The general substrate consumption rate of enzymatic hydrolysis is described as:

$$r_K = \phi_1(C_K, C_P) \cdot \phi_2(t) \quad (3)$$

Where ϕ_1 describes reaction kinetics and describes the effect of enzymatic activity on the reaction rate of hydrolysis. $\phi_1(C_K)$ is expressed with Michaelis-Menten equation by relating the reaction rate to the concentration of substrate:

$$\phi_1(C_K) = \frac{V_{max} \cdot C_K}{K_m + C_K} \quad (4)$$

where V_{max} is the maximum reaction velocity (g/l/h) and K_m is the Michaelis-Menten substrate saturation constant (g/l). To capture the decrease in reaction rate with increasing substrate conversion, non-competitive inhibition, a combination of competitive and uncompetitive inhibition affecting both V_{max} and K_m is also included in the model. The inhibition term is considered as a function of the product and substrate concentrations, representing the proteins being produced and low free water content at high solids loadings impeding the keratin-enzyme interaction. After adding the inhibition term, equation 4 becomes:

$$\phi_1(C_P, C_K) = \frac{V_{max}}{(1 + \frac{C_P}{K_I})} \cdot \frac{C_K}{K_m \cdot (1 + \frac{C_P}{K_I}) + C_K} \quad (5)$$

where, K_I is the noncompetitive inhibition constant (g/l).

As for ϕ_2 , the conventional two-parameter first order enzymatic deactivation expression was described by summation of two parallel first order reactions to capture different enzymatic deactivation rates of different enzymes in the cocktail.

$$E_A = f_A \cdot E_{tot} \quad (6)$$

$$E_f = E_{tot} - E_A \quad (7)$$

$$\phi_2(t) = E_f \cdot [e_{ia} \cdot \exp(-k_{da} \cdot t) + e_{ib} \cdot \exp(-k_{db} \cdot t)] \quad (8)$$

where, E_{tot} is the total enzyme concentration (%), E_A is the adsorbed part of the total enzyme concentration (%), E_f is the free part of the total enzyme concentration (%), f_A is the adsorption fraction, e_{ia} is the relative initial concentration of enzymes that lose activity at the reaction temperature (%), e_{ib} is the relative initial concentration of enzymes that are stable at the reaction temperature (%), k_{da} is the enzymatic decay coefficient of fast reaction and k_{db} is the enzymatic decay coefficient of slow reaction.

Parameters related to enzymatic activity (e_{ia} , e_{ib} , k_{da} , k_{db} , f_A) were directly estimated with the experimental data collected from cell-free crude keratinase extract thermal stability test and from enzyme adsorption test with 1-hour reaction time. The Michaelis-Menten parameters (V_{max} and K_m) were estimated from the hydrolysis experiment for initial kinetics, while hydrolysis yield (f) was determined from 72-hour hydrolysis experiments.. Finally, the non-competitive inhibition constant (K_I) was estimated by minimizing a defined cost function as sum squared errors

$$\min \sum (f(x_j, \theta) - y_j)^2 \quad (9)$$

where, $f(x_j, \theta)$ is the keratin and protein predictions and y_j is the corresponding experimental data.

3. Results and discussion

3.1.Enzyme stability

The experimental results are presented in Figure 1a, and the model fit at 40 °C with 95% confidence intervals is presented in Figure 1b.

Table 1. Parameters for enzymatic activity

Parameter	Unit	Value
e _{ia}	%	61.64
e _{ib}	%	38.36
k _{da}	h ⁻¹	0.19
k _{db}	h ⁻¹	5·10 ⁻⁴

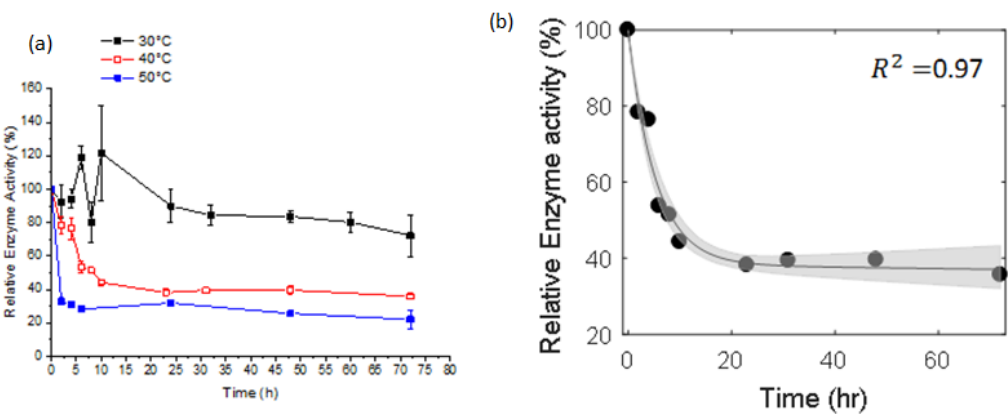


Figure 1. Enzyme stability. (a): Experimental results of cell-free crude keratinase extract thermal stability test at 30, 40 and 50 °C. (b) Model fit of enzymatic activity at 40 °C with 95% confidence intervals.

The enzymatic cocktail produced by *A. keratiniphila* D2 showed increased deactivation with increasing temperatures. An essential feature of the enzymatic cocktail was observed as the enzymatic activity is never being lost entirely within experimental time period at none of the tested temperatures (30, 40 and 50 °C). As presented in Figure 1a, deactivation stops on a level that is above 0, where the relative activity reaches a plateau. The reason of the observed phenomenon could be ex-

plained with the bacterial degradation mechanism of keratin. For instance, Yamamura et al. (2002b) suggest that bacteria produced mainly two different types of enzymes including disulfide reductases and proteases. These two types of enzymes could exert having a synergistic action on the keratinous material, i.e. one without the other cannot consume the whole keratin-rich substrate. Moreover, in this case, *A. keratiniphila* D2 produced a cocktail which contains several types of enzymes to consume the native keratin, and components of the enzymatic cocktail had different decay rate at the experimental temperatures. Thus, some types of enzymes lose their activities at low temperatures while the rest can resist higher temperatures. For all three temperatures, an initial fast deactivation is followed by a slow deactivation. Therefore, it is possible to explain the enzymatic deactivation in two phases. At the initial phase, enzymes which are affected by the reaction temperature lose their activities, and at the later phase enzymes which are stable at that temperature retain their activities. A fast deactivation was observed with high decay rates where substantial deactivation happened at the initial phase. As presented in Figure 1a, the enzymatic decay rate becomes higher and the duration of the initial phase gets shortened with higher temperatures. At the later phase, very slow deactivation happened since only small changes were observed in the enzymatic activity. Enzymatic deactivation kinetics had a biphasic behavior which could be described by two parallel first order reactions. Regarding three experimental temperatures, at 30 °C, lowest deactivation was observed where the enzymatic cocktail lost about 20% of its activity while the cocktail lost almost 62% of its activity at 40 °C. Highest deactivation was observed with approximately 73% at 50 °C.

In Figure 1b, eq. (8) was used to fit the experimental data set obtained at 40 °C and estimated parameters were used in the full model later.

3.2.Initial kinetics

The data set from the small-scale hydrolysis experiments with a reaction time of 1 hour were used to fit the parameters (V_{\max} and K_m) in the Michaelis-Menten expression (Eqs. (4)-(5)). Moreover,

the data set from enzyme adsorption test with a reaction time of 1 hour was used to determine the enzyme adsorption fraction (f_A) in eq. (8). The estimated parameters are presented in table 2, while the experimental results are presented in figure 2.

Table 2. Parameters for initial kinetics

Parameter	Unit	Value
V_{\max}	g/l/h	3.20
K_m	g/l	14.29
f_A	-	0.11

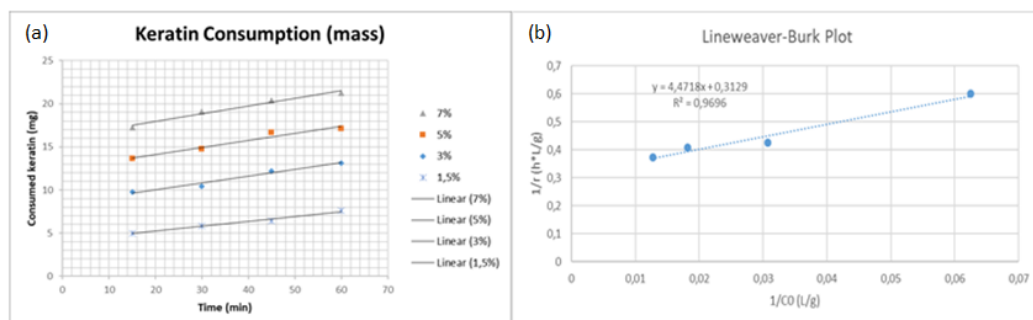


Figure 2. Initial kinetics. (a): Substrate consumption data from the small scale hydrolysis experiments at four different solids loading (1.5, 3, 5, 7 w/w %). (b): Lineweaver-Burk plot to determine the parameters of Michaelis-Menten equation.

As it can be seen from the linearity of the graphs in figure 2a, the first hour of the hydrolysis is dominated by the first-order reaction of the enzyme-substrate complex. Initial reaction rates for 1.5, 3, 5 and 7 w/w % solids loadings were determined from the slopes of the linear curves in figure 2a as 1.66, 2.353, 2.446 and 2.68 g/l/h respectively. Clearly, as the initial substrate concentration increases, the initial reaction rate of hydrolysis also increases. A linear relationship for reaction rates between different solids loadings was also found and presented in a Lineweaver-Burk plot (figure 2b) which was used to determine the parameters (V_{\max} , K_m) in the Michaelis-Menten expression.

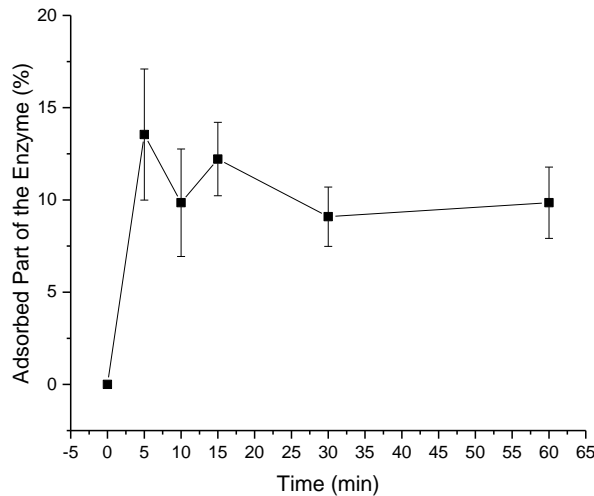


Figure 3. Percentage of the adsorbed enzyme within the first hour of hydrolysis at 40 °C and 3 w/w % solids loading.

Another important aspect of hydrolysis is the inactive part of the enzymatic cocktail. It was observed from the enzyme adsorption test (figure 3), that a small part of the cocktail is adsorbed on the substrate surface at the very beginning of the hydrolysis and is not anymore active. Thus, it means that the whole amount of the cocktail cannot take part in the hydrolysis. As it is presented in figure 3, approximately 11% of the enzyme is adsorbed on the substrate surface very fast and determined as the inactive part of the cocktail while the rest of the free part is determined as the active part in hydrolysis.

3.3. Hydrolysis and the full model

The experimental data from the small-scale batch hydrolysis experiments with a reaction time of 72 hours were used to assess the general behavior of enzymatic hydrolysis of keratin and to fit the final parameter (K_I) in the full hydrolysis model (Eqs. (1)-(3)). The estimated parameters are presented in table 3 and the hydrolysis data with 95% confidence intervals are presented in figure 4.

Table 3. Estimated parameters for noncompetitive inhibition with unique yields at different solids loadings.

	3 w/w % solids	5 w/w % solids	7 w/w % solids
Hydrolysis yield ($\text{g}_{\text{protein}}/\text{g}_{\text{keratin}}$)	0.5052	0.5821	0.6487
Noncompetitive inhibition term (K_i)	0.4636	0.6274	0.8531

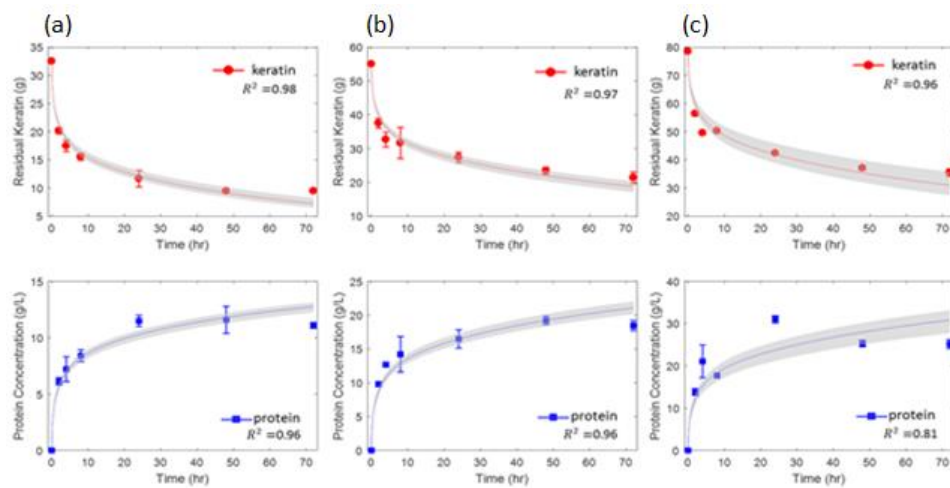


Figure 4. Residual substrate and protein production data for batch hydrolysis of keratin and its full model with 95% confidence intervals. (a): Model fit for 3 w/w % solids loading. (b): Model fit for 5 w/w % solids loading. (c): Model fit for 7 w/w % solids loading.

As it can be expected, experimental results show similar behavior for different solids loadings. According to figure 4, the hydrolysis starts very fast with a very high reaction rate within the first 4 hours at all solids loadings and the reaction rate starts to decrease considerably following the 4 and 24-hour. Hydrolysis almost stops after 24 hours but it continues very slowly with only minor changes in protein production and substrate consumption afterwards. In other words, reaction rate decreases with respect to substrate conversion, and keratin consumption and protein production is being stagnated after 24 hours despite of the presence of active enzymes. Reasons behind this phenomenon are not fully known however some speculations can be made about the reasons.

The first reason might be the decrease in the specific activity of the adsorbed enzyme. Results from the hydrolysis experiment showed good agreement with the enzyme stability test since the major part of the enzymatic cocktail loses its activity after 10 hours which corresponds to the time point where the reaction rate of the hydrolysis considerably starts to decrease. Keratin degradation mechanism by keratinases is still a matter of debate. It was shown that keratinase alone could bring about complete degradation of raw feather (Lin et al., 1992), whereas other studies showed that keratin degradation was initiated and assisted by the disulfide reductase enzyme (Ramnani et al., 2005). In the latter case, bacterial degradation of native keratin involves reduction of disulfide bonds and enzymatic decomposition of keratin proteins. Bacteria produce two types of enzymes; one is protein-disulfide reductase and the other type is serine protease. At the first stage of bacterial degradation of native keratin, protein-disulfide reductase takes place and breaks the disulfide bonds to denature the keratin structure and make it available for enzymatic lysis. At the second stage, protease decomposes keratin and releases amino acids and peptides. To degrade keratin, those two types of enzymes have to work together. As it was stated before, the enzyme produced by *A. keratiniphila* D2 which was used in the hydrolysis experiments is a cocktail that contains different types of enzymes. Unfortunately, the exact composition of the cocktail is not known. The presence of different types of enzymes might affect the others specific activities.

The second reason might be related to speculations about the presence or not of easily hydrolysable and non-degradable fractions of the substrate. In the hydrolysis of cellulose, a reaction system known to have many parallels to keratin hydrolysis (Lange et al., 2016), it is a well-known fact that cellulose can be classified into easily and difficult hydrolysable cellulose and from the empirical data it is possible to estimate their fractions (Sattler et al., 1989). Also, it may be the case in this study. In cellulose studies, it is observed that easily and difficult hydrolysable parts show a variation

related to enzymatic activity (Sattler et al., 1989). Also, here it could be possible to classify keratin as readily and slowly hydrolysable.

Regarding the three different solids loadings, higher substrate consumption and higher protein production was observed at higher solids loadings. However, when the consumed amount of substrate is compared to the total amount, it was observed that the hydrolyzed fraction of the whole substrate decreases with higher solids loadings. 23.04, 33.62 and 43.05 g/l of substrate corresponding to 70.87, 61.12 and 54.84% of the total amount of substrate were consumed at 3, 5 and 7 w/w % solids loadings respectively. This shows that the enzyme cocktail displays a poorer ability to degrade keratin at higher solids loadings. Regarding hydrolysis, negligible factors in low-solids loadings become more prominent in high-solids loadings condition (Modenbach and Nokes, 2013). First of all, there could be larger mass transfer limitations with increased solids loadings. The free water content is very important to perform effective hydrolysis since it has a significant role to play in mass transfer and lubricity. Presence of water increases the effectiveness of enzymatic reactions by providing a medium for solubilizing and aiding in the mass transfer of products. At the same time, as the free water content of the medium increases, the lubricity of particles also increases. A low amount of free water causes high viscosity that makes mixing and handling of the material more difficult. Thus, also in this study, it is possible that mass transfer between the keratin and enzyme is becoming impeded at high substrate concentrations, due to reduced free water content as the keratin holds the water more tightly within its pores than the gravitational force acts on it. This situation is limiting the diffusion of products away from the enzyme. Moreover, the inhibition effect is not only caused by products, but also caused by released inhibitory compounds from the biomass. Therefore, limited diffusion of such products away from the enzyme may cause formation of an undesired product-enzyme complex instead of a substrate-enzyme complex, which decreases effectiveness of the enzyme and results in a higher inhibition factor as presented in table 4.

Normally, it is expected to have fixed yields independently from initial substrate concentrations. However, in this study, increasing yields at higher solids loadings have been observed. The yields were calculated as 0.48, 0.55, 0.58 $\text{g}_{\text{protein}}/\text{g}_{\text{keratin}}$ for 3, 5 and 7 w/w % solids loadings respectively. The lowest yield was observed when the highest proportion of substrate is consumed and vice versa. The cause might be caused by further breakdown of soluble proteins to polypeptides and amino acids by some proteases in the enzymatic cocktail which cannot be captured by the BCA protein assay. Additionally, it should be noted that with keratin degradation, not only soluble proteins are released but also structural substances such as lipids are also being co-extracted together with the proteic part and the BCA protein assay is not able to detect those substances. Nevertheless, as the fraction of consumed substrate increases, release of those structural substances and further breakdown of soluble proteins increase which lead to small changes in yields.

Figure 4 presents the experimental state data and the model fit and the standard deviation of triplicate samples over 72 hours of hydrolysis for three initial substrate concentrations. A good agreement between experimental data and model fit can be observed for both substrate consumption and protein production data at all solids loadings. These observations suggest that the proposed model for enzymatic hydrolysis of keratinous substrate is able to capture the key behavior that was observed in the experiments. The only poor fitting can be observed for protein production at 7 w/w % solids loading (Fig. 4c). Two outlier data points at 4 and 24-hour time points in this experimental set can be identified as the reason of poor fitting at this solids loading. Due to the different yields, in full model, model fitting was performed individually for each solids loading by defining different yields uniquely and the inhibition term (K_i) was estimated separately for each solids loading. Parameter estimation shows that both inhibition coefficient and hydrolysis yield increase together with increased solids loading. This might show that there is a correlation between the inhibition constant and hydrolysis yield, that results in a model parameter identification problem. This problem is quite

common when performing parameter estimation for this type of processes (Tervasmäki et al., 2017). Thus, it is very important to find the mechanism behind larger hydrolysis yield with increased initial substrate concentration and a possible mathematical relationship between them. If the mechanism can be incorporated into an updated model it would be of even more value for accurately representing the system dynamics.

The model can successfully capture the reaction rate within 24 hours; however it is not very accurate in capturing the low reaction rate which starts to be seen after 24 hours. Following this point in time, the reaction rate in the model also gets lower, however it is not as low as in the actual hydrolysis and it takes longer for the model to reach the point where keratin consumption and protein production reach a plateau.

4. Conclusions

The model describes the enzymatic hydrolysis of keratin with the keratinophilic enzymatic cocktail produced by *A. keratiniphila* D2. Enzymatic activity, Michaelis-Menten kinetics and non-competitive inhibition are used to capture the observed behavior of the hydrolysis when decreasing reaction rate were observed with increasing substrate conversion. Different solids loadings have been discretized in modelling to apply different hydrolysis yields observed at different initial substrate concentrations. The proposed model is suitable to be used as a base to understand the keratin hydrolysis process and to be used in further improvement and optimization studies. Further experimental work is suggested to increase the accuracy in an updated model by identification of exact composition of the enzymatic cocktail and confirmation of the speculations on the different hydrolysable fractions of the keratin for different solids loadings and mass transfer limitations. Also, investigation of the temperature dependency of the parameters and effects of different enzyme dosages to hydrolysis would be of great value to increase model accuracy for application towards dynamical optimization of the keratin hydrolysis reactor.

References:

1. Abdul-Hamid, A., Bakar, J., Bee, G.H., 2002. Nutritional quality of spray dried protein hydrolysate from Black Tilapia (*Oreochromis mossambicus*). Food Chem. 78, 69–74.
2. Al-Mussallam, A.A., Al-Zarban, S.S., Fasasi, Y.A., Kroppenstedt, R.M., Stackebrandt, E., 2003. *Amycolatopsis keratiniphilia* sp. nov., a novel keratinolytic soil actinomycete from Kuwait. Int. J. Syst. Evol. Microbiol. 53, 871–874.
3. Al-Mussallam, A.A., Al-Zarban, S.S., Al-Sarawi, H., Fasasi, Y.A., Kroppenstedt, R.M., Stackebrandt, E., 2003. Influence of nutritional supplements on keratinolysis by *Amycolatopsis keratiniphila*. Indian J. Exp. Biol. 4, 870–874.
4. Anbu, P., Hilda, A., Sur, H.W., Hur, B.K., Jayanthi, S., 2008. Extracellular keratinase from *Trichophyton* sp. HA-2 isolated from feather dumping soil. Int. Biodeterior. Biodegrad. 62, 287–292.
5. Apodaca, G., McKerrow, J.H., 1990. Expression of proteolytic activity by cultures of *Trichophyton rubrum*. J. Med. Vet. Mycol. 28, 159–71.
6. Awad, G.E.A., Esawy, M.A., Salam, W.A., Salama, B.M., Abdelkader, A.F., El-diwany, A., 2011. Keratinase production by *Bacillus pumilus* GHD in solid-state fermentation using sugar cane bagasse: optimisation of culture conditions using a Box-Behnken experimental design. Ann. Microbiol. 61, 663–672.
7. Baldo, A., Tabart, J., Vermout, S., Mathy, A., Collard, A., Losson, B., Mignon, B., 2008. Secreted subtilisins of *Microsporium canis* are involved in adherence of arthroconidia to feline corneocytes. J. Med. Microbiol. 57, 1152–1156.
8. Benesova, P., Kucera, D., Marova, I., Obruca, S., 2017. Chicken feather hydrolysate as an inexpensive complex nitrogen source for PHA production by *Cupriavidus necator* on waste frying oils. Lett. Appl. Microbiol. 65, 182–188.
9. Benkiar, A., Nadia, Z.J., Badis, A., Rebzani, F., Soraya, B.T., Rekik, H., Naili, B., Ferradji, F.Z., Bejar, S., Jaouadi, B., 2013. Biochemical and molecular characterization of a thermo- and detergent-stable alkaline serine keratinolytic protease from *Bacillus circulans* strain DZ100 for detergent formulations and feather-biodegradation process. Int. Biodeterior. Biodegrad. 83, 129–138.
10. Bertsch, A., Coello, N., 2005. A biotechnological process for treatment and recycling poultry feathers as a feed ingredient. Bioresour. Technol. 96, 1703–1708.

11. Bhavsar, P.S., Zoccola, M., Patrucco, A., Montarsolo, A., Mossotti, R., Giansetti, M., Rovero, G., Maier, S.S., Muresan, A., Tonin, C., 2017. Superheated Water Hydrolyzed Keratin: A New Application as a Foaming Agent in Foam Dyeing of Cotton and Wool Fabrics. *ACS Sustain. Chem. Eng.* 5, 9150–9159.
12. Biggs, A.I., 1954. A spectrophotometric determination of the dissociation constants of p-nitrophenol and papaverine. *Trans. Faraday Soc.* 50, 800.
13. Bockle, B., Galunsky, B., Muller, R., 1995. Characterization of a keratinolytic serine proteinase from *Streptomyces pactum* DSM40530. *Appl. Environ. Microbiol.* 61, 3705–3710.
14. Bockle, B., Muller, R., 1997. Reduction of disulfide bonds by *Streptomyces pactum* during growth on chicken feathers. *Appl. Environ. Microbiol.* 63, 790–792.
15. Bohacz, J., 2017. Biodegradation of feather waste keratin by a keratinolytic soil fungus of the genus *Chrysosporium* and statistical optimization of feather mass loss. *World J. Microbiol. Biotechnol.* 33.
16. Bouacem, K., Bouanane-Darenfed, A., Zarái Jaouadi, N., Joseph, M., Hacene, H., Ollivier, B., Fardeau, M.L., Bejar, S., Jaouadi, B., 2016. Novel serine keratinase from *Caldicoprobacter algeriensis* exhibiting outstanding hide dehairing abilities. *Int. J. Biol. Macromol.* 86, 321–328.
17. Bougatef, A., Nedjar-Arroume, N., Manni, L., Ravallec, R., Barkia, A., Guillochon, D., Nasri, M., 2010. Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (*Sardinella aurita*) by-products proteins. *Food Chem.* 118, 559–565.
18. Bragulla, H.H., Homberger, D.G., 2009. Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia. *J. Anat.* 214, 516–559.
19. Brandelli, A., 2008. Bacterial keratinases: Useful enzymes for bioprocessing agroindustrial wastes and beyond. *Food Bioprocess Technol.* 1, 105–116.
20. Brandelli, A., Daroit, D.J., Riffel, A., 2010. Biochemical features of microbial keratinases and their production and applications. *Appl. Microbiol. Biotechnol.* 85, 1735–1750.
21. Brandelli, A., Sala, L., Kalil, S.J., 2015. Microbial enzymes for bioconversion of poultry waste into added-value products. *Food Res. Int.* 73, 3–12.
22. Brouta, F., Descamps, F., Monod, M., Losson, B., Mignon, B., Vermout, S., 2002. Secreted Metalloprotease Gene Family of *Microsporum canis*. *Infect. Immun.* 70, 5676–5683.
23. Buck, F.F., Bier, M., Nord, F.F., 1962a. Some properties of human trypsin. *Arch. Biochem. Biophys.* 98, 528–530.

24. Buck, F.F., Vithayathil, A.J., Bier, M., Nord, F.F., 1962b. On the mechanism of enzyme action. LXXIII. Studies on trypsins from beef, sheep and pig pancreas. Arch. Biochem. Biophys. 97, 417–424.
25. Burg, J.M., Cooper, C.B., Ye, Z., Reed, B.R., Moreb, E.A., Lynch, M.D., 2016. Large-scale bioprocess competitiveness: the potential of dynamic metabolic control in two-stage fermentations. Curr Opin Chem Eng. 14, 121–136.
26. Cai, C., Zheng, X., 2009. Medium optimization for keratinase production in hair substrate by a new *Bacillus subtilis* KD-N2 using response surface methodology. J. Ind. Microbiol. Biotechnol. 36, 875–883.
27. Casarin, F., Cladera-Olivera, F., Brandelli, A., 2008. Use of Poultry Byproduct for Production of Keratinolytic Enzymes. Food Bioprocess Technol. 1, 301–305.
28. Chen, X., Zhou, B., Xu, M., Huang, Zhiqing., Jia, G., Zhao, H., Liu, G., 2015. Prokaryotic expression and characterization of a keratinolytic protease from *Aspergillus niger*. Biologia. 70, 157–164.
29. Cheng, S., Hu, H., Shen, S., Takagi, H., Asano, M., Tsai, Y., 1995. Production and Characterization of Keratinase of a Feather-degrading *Bacillus licheniformis* PWD-I. Biosci. Biotech. Biochem. 59, 2239–2243.
30. Chojnacka, K., Górecka, H., Michalak, I., Górecki, H., 2011. A review: Valorization of keratinous materials. Waste and Biomass Valorization 2, 317–321.
31. Contesini, F.J., Melo, R.R. de, Sato, H.H., 2018. An overview of *Bacillus* proteases: from production to application. Crit. Rev. Biotechnol. 38, 321–334.
32. Da Gioppo, N.M.R., Moreira-Gasparin, F.G., Costa, A.M., Alexandrino, A.M., De Souza, C.G.M., Peralta, R.M., 2009. Influence of the carbon and nitrogen sources on keratinase production by *Myrothecium verrucaria* in submerged and solid state cultures. J. Ind. Microbiol. Biotechnol. 36, 705–711.
33. Daroit, D.J., Brandelli, A., 2014. A current assessment on the production of bacterial keratinases. Crit. Rev. Biotechnol. 34, 372–384.
34. Daroit, D.J., Corrêa, A.P.F., Brandelli, A., 2009. Keratinolytic potential of a novel *Bacillus* sp. P45 isolated from the Amazon basin fish *Piaractus mesopotamicus*. Int. Biodeterior. Biodegradation. 63, 358–363.

35. Daroit, D.J., Corrêa, A.P.F., Brandelli, A., 2011. Production of keratinolytic proteases through bioconversion of feather meal by the Amazonian bacterium *Bacillus* sp. P45. *Int. Biodeterior. Biodegradation*. 65, 45–51.
36. Demidyuk, I.V., Chukhontseva, K.N., Kostrov, S.V., 2017. Glutamyl Endopeptidases: The Puzzle of Substrate Specificity. *Acta Naturae*. 9, 17–33.
37. Demir, T., Hameş, E.E., Öncel, S.S., Vardar-Sukan, F., 2015. An optimization approach to scale up keratinase production by *Streptomyces* sp. 2M21 by utilizing chicken feather. *Int. Biodeterior. Biodegrad*. 103, 134–140.
38. Deng, Y.P., Jiang, W.D., Liu, Y., Jiang, J., Kuang, S.Y., Tang, L., Wu, Z., Zhang, Y.A., Feng, L., Zhou, X.Q., 2014. Differential growth performance, intestinal antioxidant status and relative expression of Nrf2 and its target genes in young grass carp (*Ctenopharyngodon idella*) fed with graded levels of leucine. *Aquaculture* 434, 66–73.
39. Dou, Y., Zhang, B., He, M., Yin, G., Cui, Y., 2016. The structure, tensile properties and water re-sistance of hydrolyzed feather keratin-based bioplastics. *Chinese J. Chem. Eng.* 24, 415–420.
40. Ebeling, W., Hennrich, N., Klockow, M., Metz, H., Orth, H.D., Lang, H., 1974. Proteinase K from *Tritirachium album* Limber. *Eur. J. Biochem.* 47, 91–97.
41. El-Gendy, M.M.A., 2010. Keratinase Production by Endophytic *Penicillium* spp. Morsyl Under Solid-State Fermentation Using Rice Straw. *Appl. Biochem. Biotechnol.* 162, 780–794.
42. El-Refai, H.A., AbdelNaby, M.A., Gaballa, A., El-Araby, M.H., Abdel Fattah, A.F., 2005. Improvement of the newly isolated *Bacillus pumilus* FH9 keratinolytic activity. *Process Biochem.* 40, 2325–2332.
43. Embaby, A.M., Zaghloul, T.I., Elmahdy, A.R., 2010. Optimizing the biodegradation of two keratinous wastes through a *Bacillus subtilis* recombinant strain using a response surface methodology. *Biodegradation* 21, 1077–1092.
44. Esteban, M.B., García, A.J., Ramos, P., Márquez, M.C., 2010. Sub-critical water hydrolysis of hog hair for amino acid production. *Bioresour. Technol.* 101, 2472–2476.
45. Fakhfakh, N., Ktari, N., Haddar, A., Mnif, I.H., Dahmen, I., Nasri, M., 2011. Total solubilisation of the chicken feathers by fermentation with a keratinolytic bacterium, *Bacillus pumilus* A1, and the production of protein hydrolysate with high antioxidative activity. *Process Biochem.* 46, 1731–1737.

46. Fakhfakh, N., Ktari, N., Siala, R., Nasri, M., 2013. Wool-waste valorization: production of protein hydrolysate with high antioxidative potential by fermentation with a new keratinolytic bacterium, *Bacillus pumilus* A1. J. Appl. Microbiol. 115, 424–433.
47. Fakhfakh-Zouari, N., Haddar, A., Hmidet, N., Frikha, F., Nasri, M., 2010. Application of statistical experimental design for optimization of keratinases production by *Bacillus pumilus* A1 grown on chicken feather and some biochemical properties. Process Biochem. 45, 617–626.
48. Fan, J., Liu, J.F., He, J.H., 2008. Hierarchy of wool fibers and fractal dimensions. International Journal of Nonlinear Sciences and Numerical Simulation. 9, 293–296.
49. Fang, Z., Zhang, J., Liu, B., Du, G., Chen, J., 2013a. Biochemical characterization of three keratinolytic enzymes from *Stenotrophomonas maltophilia* BBE11-1 for biodegrading keratin wastes. Int. Biodeter. Biodegr. 82, 166–172.
50. Fang, Z., Zhang, J., Liu, B., Du, G., Chen, J., 2013b. Biodegradation of wool waste and keratinase production in scale-up fermenter with different strategies by *Stenotrophomonas maltophilia* BBE11-1. Bioresour. Technol. 140, 286–291.
51. Fang, Z., Zhang, J., Liu, B., Jiang, L., Du, G., Chen, J., 2014. Cloning, heterologous expression and characterization of two keratinases from *Stenotrophomonas maltophilia* BBE11-1. Process Biochem. 49, 647–654.
52. Farag, A.M., Hassan, M.A., 2004. Purification, characterization and immobilization of a keratinase from *Aspergillus oryzae*. Enzyme Microb. Technol. 34, 85–93.
53. Fontoura, R., Daroit, D.J., Correa, A.P.F., Meira, S.M.M., Mosquera, M., Brandelli, A., 2014. Production of feather hydrolysates with antioxidant, angiotensin-I converting enzyme- and dipeptidyl peptidase-IV-inhibitory activities. N. Biotechnol. 31, 506–513.
54. Fraser, R.D.B., MacRae, T.P., Rogers, G.E., 1962. Molecular organization in alpha-keratin. Nature. 193, 1052–1055.
55. Fraser, R.D.B., MacRae, T.P., Sparrow, L.G., Parry, D.A.D., 1988. Disulfide bonding in α -keratin. Int J Biol Macromol. 10, 106–112.
56. Friedrich, A.B., Antranikian, G., 1996. Keratin degradation by *Fervidobacterium pennavorans*, a novel thermophilic anaerobic species of the order *Thermotogales*. Appl. Environ. Microbiol. 62, 2875–2882.
57. Gachango, F.G., Ekmann, K.S., Frørup, J., Pedersen, S.M., 2017. Use of pig by-products (bristles and hooves) as alternative protein raw material in fish feed: A feasibility study. Aquaculture 479, 265–272.

58. Gehle, M.H., Speers, G.M., Miller, D.L., Balloun, S.L., Economics, H., Station, E., 1967. Nutritive Value of Hydrolyzed Hog Hair as a Protein Source for Chicks and Poults. *Poult. Sci.* 46, 156–164.
59. Geng, W., Jin, Y., Jameel, H., Park, S., 2015. Strategies to achieve high-solids enzymatic hydrolysis of dilute-acid pretreated corn stover. *Bioresour. Technol.* 187, 43–48.
60. Gessesse, A., Hatti-Kaul, R., Gashe, B.A., Mattiasson, B., 2003. Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. *Enzyme Microb. Technol.* 32, 519–524.
61. Ghosh, A., Chakrabarti, K., Chattopadhyay, D., 2008. Degradation of raw feather by a novel high molecular weight extracellular protease from newly isolated *Bacillus cereus* DCUW. *J. Ind. Microbiol. Biotechnol.* 35, 825–834.
62. Gioppo, N.M.R., Moreira-Gasparin, F.G., Costa, A.M., Alexandrino, A.M., De Souza, C.G.M., Peralta, R.M., 2009. Influence of the carbon and nitrogen sources on keratinase production by *Myrothecium verrucaria* in submerged and solid state cultures. *J. Ind. Microbiol. Biotechnol.* 36, 705–711.
63. Gong, J., Wang, Y., Zhang, D., Zhang, R., Su, C., Li, H., Zhang, X., Xu, Z., Shi, J., 2015. Biochemical characterization of an extreme alkaline and surfactant-stable keratinase derived from a newly isolated actinomycete *Streptomyces aureofaciens* K13. *RSC Adv.* 5, 24691–24699.
64. Gonzalez-Silvera, D., Herrera, M., Giráldez, I., Esteban, M., 2018. Effects of the Dietary Tryptophan and Aspartate on the Immune Response of Meagre (*Argyrosomus regius*) after Stress. *Fishes* 3, 6.
65. Govarthanan, M., Selvankumar, T., Selvam, K., Sudhakar, C., Aroulmoji, V., Kamala-Kannan, S., 2015. Response surface methodology based optimization of keratinase production from alkali-treated feather waste and horn waste using *Bacillus* sp. MG-MASC-BT. *J. Ind. Eng. Chem.* 27, 25–30.
66. Gradišar, H., Friedrich, J., Križaj, I., Jerala, R., 2005. Similarities and specificities of fungal keratinolytic proteases: Comparison of keratinases of *Paecilomyces marquandii* and *Doratomyces microsporus* to some known proteases. *Appl. Environ. Microbiol.* 71, 3420–3426.
67. Graham, C., Waitkoff, H., Hier, S., 1949. The amino acid content of some scleroproteins. *J. Biol. Chem.* 177, 529–532.
68. Grazziotin, A., Pimentel, F.A., de Jong, E.V., Brandelli, A., 2006. Nutritional improvement of feather protein by treatment with microbial keratinase. *Anim. Feed Sci. Technol.* 126, 135–144.

69. Grazziotin, A., Pimentel, F.A., Jong, E.V. De, Brandelli, A., 2008. Poultry feather hydrolysate as a protein source for growing rats. *Brazilian J. Vet. Res. Anim. Sci.* 45, 61–67.
70. Grazziotin, A., Pimentel, F.A., Sangali, S., de Jong, E. V., Brandelli, A., 2007. Production of feather protein hydrolysate by keratinolytic bacterium *Vibrio sp.* kr2. *Bioresour. Technol.* 98, 3172–3175.
71. Gupta, N., Hixson, K.K., Culley, D.E., Smith, R.D., Pevzner, P.A., 2010. Analyzing protease specificity and detecting in vivo proteolytic events using tandem mass spectrometry. *Proteomics* 10, 2833–2844.
72. Gupta, R., Beg, Q.K., Khan, S., Chauhan, B., 2002. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Appl. Microbiol. Biotechnol.* 60, 381–395.
73. Gupta, R., Ramnani, P., 2006. Microbial keratinases and their prospective applications: an overview. *Appl. Microbiol. Biotechnol.* 70, 21–33.
74. Gupta, R., Sharma, R., Beg, Q.K., 2013. Revisiting microbial keratinases: next generation proteases for sustainable biotechnology. *Crit. Rev. Biotechnol.* 33, 216–228.
75. Habbeche, A., Saoudi, B., Jaouadi, B., Haberra, S., Kerouaz, B., Boudelaa, M., Badis, A., Ladjama, A., 2014. Purification and biochemical characterization of a detergent-stable keratinase from a newly thermophilic actinomycete *Actinomadura keratinilytica* strain Cpt29 isolated from poultry compost. *J. Biosci. Bioeng.* 117, 413–421.
76. Hamre, K., Kolås, K., Sandnes, K., 2010. Protection of fish feed, made directly from marine raw materials, with natural antioxidants. *Food Chem.* 119, 270–278.
77. Han, A.-R., Kang, H.-R., Son, J., Kwon, D.H., Kim, S., Lee, W.C., Song, H.K., Song, M.J., Hwang, K.Y., 2016. The structure of the pleiotropic transcription regulator CodY provides insight into its GTP-sensing mechanism. *Nucleic Acids Res.* 44, 9483–9493.
78. Hertrampf, J.W., Piedad-Pascual, F., 2000a. Feather Meal (Hydrolysed), in: *Handbook on Ingredients for Aquaculture Feeds*. Springer Netherlands, Dordrecht, pp. 131–141.
79. Hertrampf, J.W., Piedad-Pascual, F., 2000b. Pig Bristle Meal (Hydrolysed), in: *Handbook on Ingredients for Aquaculture Feeds*. Springer Netherlands, Dordrecht, pp. 322–324.
80. Hodge, D.B., Karim, M.N., Schell, D.J., McMillan, J.D., 2009. Model-based fed-batch for high-solids enzymatic cellulose hydrolysis. *Appl. Biochem. Biotechnol.* 152, 88–107.

81. Hou, Y., Wu, Z., Dai, Z., Wang, G., Wu, G., 2017. Protein hydrolysates in animal nutrition: Industrial production, bioactive peptides, and functional significance. *J. Anim. Sci. Biotechnol.* 8, 24.
82. Hu, H., Gao, J., He, J., Yu, B., Zheng, P., Huang, Z., Mao, X., Yu, J., Han, G., Chen, D., 2013b. Codon Optimization Significantly Improves the Expression Level of a Keratinase Gene in *Pichia pastoris*. *PLoS One* 8, e58393.
83. Hu, H., He, J., Yu, B., Zheng, P., Huang, Z., Mao, X., Yu, J., Han, G., Chen, D., 2013a. Expression of a keratinase (kerA) gene from *Bacillus licheniformis* in *Escherichia coli* and characterization of the recombinant enzymes. *Biotechnol. Lett.* 35, 239–244.
84. Huang, Y., Busk, P.K., Herbst, F.-A., Lange, L., 2015. Genome and secretome analyses provide insights into keratin decomposition by novel proteases from the non-pathogenic fungus *Onygena corvina*. *Appl. Microbiol. Biotechnol.* 99, 9635–9649.
85. Jaouadi, B., Abdelmalek, B., Fodil, D., Ferradji, F.Z., Rekik, H., Zeraï, N., Bejar, S., 2010. Purification and characterization of a thermostable keratinolytic serine alkaline proteinase from *Streptomyces* sp. strain AB1 with high stability in organic solvents. *Bioresour. Technol.* 101, 8361–8369.
86. Jin, E., Reddy, N., Zhu, Z., Yang, Y., 2011. Graft Polymerization of Native Chicken Feathers for Thermoplastic Applications. *J. Agric. Food Chem.* 59, 1729–1738.
87. Jones, L.N., Rivett, D.E., Tucker, D.J., 2006. Wool and related mammalian fibers, in: Lewin, M. (Eds.), *Handbook of Fiber Chemistry*. third ed. CRC Press, Boca Raton, pp. 367–399.
88. Joo, H.-S., Chang, C.-S., 2005. Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: optimization and some properties. *Process Biochem.* 40, 1263–1270.
89. Jos, A., Cameán, A.M., Pflugmacher, S., Segner, H., 2009. The antioxidant glutathione in the fish cell lines EPC and BCF-2: Response to model pro-oxidants as measured by three different fluorescent dyes. *Toxicol. Vitro* 23, 546–553.
90. Joshi, R.H., Dodia, M.S., Singh, S.P., 2008. Production and optimization of a commercially viable alkaline protease from a haloalkaliphilic bacterium. *Biotechnol. Bioprocess Eng.* 13, 552–559.
91. Kelstrup, C.D., Young, C., Lavalley, R., Nielsen, M.L., Olsen, J. V., 2012. Optimized fast and sensitive acquisition methods for shotgun proteomics on a quadrupole orbitrap mass spectrometer. *J. Proteome Res.* 11, 3487–3497.

92. Klamt, S., Mahadevan, R., Hädicke, O., 2017. When do two-stage processes outperform one-stage processes? *Biotechnol J. Nov* 13. doi: 10.1002/biot.201700539.
93. Kornilłowicz-Kowalska, T., Bohacz, J., 2011. Biodegradation of keratin waste: Theory and practical aspects. *Waste Manag.* 31, 1689–1701.
94. Kothari, D., Rani, A., Goyal, A., 2017. Keratinases, in: Pandey, A., Negi, S., Soccol, C.R. (Eds.), *Current Developments in Biotechnology and Bioengineering. Production, Isolation and Purification of Industrial Products*. Elsevier Science, pp. 447–469.
95. Kristensen, J.B., Felby, C., Jørgensen, H., 2009. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. *Biotechnol. Biofuels.* 2, 11.
96. Kristinsson, H.G., Rasco, B.A., 2000. Biochemical and functional properties of Atlantic salmon (*Salmo salar*) muscle proteins hydrolyzed with various alkaline proteases. *J. Agric. Food Chem.* 48, 657–666.
97. Kshetri, P., Roy, S.S., Sharma, S.K., Singh, T.S., Ansari, M.A., Prakash, N., Ngachan, S. V., 2017. Transforming Chicken Feather Waste into Feather Protein Hydrolysate Using a Newly Isolated Multifaceted Keratinolytic Bacterium *Chryseobacterium sediminis* RCM-SSR-7. *Waste and Biomass Valorization* 0, 1–11.
98. Kumar, A.G., Swarnalatha, S., Gayathri, S., Nagesh, N., Sekaran, G., 2008. Characterization of an alkaline active - Thiol forming extracellular serine keratinase by the newly isolated *Bacillus pumilus*. *J. Appl. Microbiol.* 104, 411–419.
99. Kumar, R., Balaji, S., Uma, T.S., Mandal, A.B., Sehgal, P.K., 2010. Optimization of influential parameters for extracellular keratinase production by *Bacillus subtilis* (MTCC9102) in solid state fermentation using horn meal-A biowaste management. *Appl. Biochem. Biotechnol.* 160, 30–39.
100. Kunert, J., 1972a. Thiosulphate esters in keratin attacked by dermatophytes *in vitro*. *Sabouraudia.* 10, 6–13.
101. Kunert, J., 1972b. Keratin decomposition by dermatophytes: evidence of the sulphitolysis of the protein. *Experientia.* 28, 1025–1026.
102. Kunert, J., 1973. Keratin decomposition by dermatophytes. I. Sulfite production as a possible way of substrate denaturation. *Z Allg Mikrobiol.* 13, 489–498.
103. Kunert, J., 1976. Keratin decomposition by dermatophytes II. Presence of S-sulfocysteine and cysteic acid in soluble decomposition products. *Zeitschrift Für Allgemeine Mikrobiologie* 2, 97–105.

104. Kunert, J., 1989. Biochemical mechanism of keratin degradation by the actinomycete *Streptomyces fradiae* and the fungus *Microsporium gypseum*: A comparison. J. Basic Microbiol. 29, 597–604.
105. Łaba, W., Choińska, A., Rodziewicz, A., 2013. The release of sulfur compounds during degradation of feather keratin by two *Bacillus* strains. Acta Sci. Pol., Biotechnologia 12, 29–40.
106. Łaba, W., Choińska, A., Rodziewicz, A., Piegza, M., 2015b. Keratinolytic abilities of *Micrococcus luteus* from poultry waste. Braz. J. Microbiol. 46, 691–700.
107. Łaba, W., Chorążyk, D., Pudło, A., Trojan-Piegza, J., Piegza, M., Kancelista, A., Kurzawa, A., Żuk, I., Kopeć, W., 2017. Enzymatic Degradation of Pretreated Pig Bristles with Crude Keratinase of *Bacillus cereus* PCM 2849. Waste and Biomass Valorization 8, 527–537.
108. Łaba, W., Kopeć, W., Chorążyk, D., Kancelista, A., Piegza, M., Malik, K., 2015a. Biodegradation of pretreated pig bristles by *Bacillus cereus* B5esz. Int. Biodeterior. Biodegradation 100, 116–123.
109. Łaba, W., Kopeć, W., Chorążyk, D., Kancelista, A., Piegza, M., Malik, K., 2015. Biodegradation of pretreated pig bristles by *Bacillus cereus* B5esz. Int. Biodeterior. Biodegradation 100, 116–123.
110. Łaba, W., Piegza, M., Kawa-Rygielska, J., 2017. Evaluation of brewer's spent grain as a substrate for production of hydrolytic enzymes by keratinolytic bacteria. J. Chem. Technol. Biotechnol. 92, 1389–1396.
111. Łaba, W., Żarowska, B., Chorążyk, D., Pudło, A., Piegza, M., Kancelista, A., Kopeć, W., 2018. New keratinolytic bacteria in valorization of chicken feather waste. AMB Express 8, 9.
112. Lall, S.P., Dumas, A., 2015. Nutritional requirements of cultured fish. Feed Feed. Pract. Aquac. 53–109.
113. Lange, L., Huang, Y., Busk, P.K., 2016. Microbial decomposition of keratin in nature-a new hypothesis of industrial relevance. Appl. Microbiol. Biotechnol. 100, 2083–2096.
114. Lansard, M., Panserat, S., Plagnes-Juan, E., Dias, K., Seiliez, I., Skiba-Cassy, S., 2011. L-Leucine, L-methionine, and L-lysine are involved in the regulation of inter-diary metabolism-related gene expression in rainbow trout hepatocytes. J. Nutr. 141, 75–80.
115. Lansard, M., Panserat, S., Plagnes-Juan, E., Seiliez, I., Skiba-Cassy, S., 2010. Integration of insulin and amino acid signals that regulate hepatic metabolism-related gene expression in rainbow trout: role of TOR. Amino Acids 39, 801–810.

116. Latshaw, J.D., Musharaf, N., Retrum, R., 1994. Processing of feather meal to maximize its nutritional value for poultry. *Anim. Feed Sci. Technol.* 47, 179–188.
117. Lemes, A.C., Álvares, G.T., Egea, M.B., Brandelli, A., Kalil, S.J., 2016. Simultaneous production of proteases and antioxidant compounds from agro-industrial by-products. *Bioresour. Technol.* 222, 210–216.
118. Li, P., Mai, K., Trushenski, J., Wu, G., 2009. New developments in fish amino acid nutrition: Towards functional and environmentally oriented aquafeeds. *Amino Acids* 37, 43–53.
119. Lin, X., Lee, C.G., Casale, E.S., Shih, J.C., 1992. Purification and Characterization of a Keratinase from a Feather-Degrading *Bacillus licheniformis* Strain. *Appl. Environ. Microbiol.* 58, 3271–5.
120. Lin, X., Wong, S.L., Miller, E.S., Shih, J.C., 1997. Expression of the *Bacillus licheniformis* PWD-1 keratinase gene in *B. subtilis*. *J. Ind. Microbiol. Biotechnol.* 19, 134–138.
121. Maciel, J.L., Werlang, P.O., Daroit, D.J., Brandelli, A., 2017. Characterization of Protein-Rich Hydrolysates Produced Through Microbial Conversion of Waste Feathers. *Waste and Biomass Valorization* 8, 1177–1186.
122. Mahan, D.C., Shields, R.G., 1998. Essential and Nonessential Amino Acid Composition of Pigs from Birth to 145 Kilograms of Body Weight, and Comparison to Other Studies. *J. Anim. Sci.* 76, 513–521.
123. Mahro, B., Timm, M., 2007. Potential of biowaste from the food industry as a biomass resource. *Eng. Life Sci.* 7, 457–468.
124. Manczinger, L., Rozs, M., Vágvölgyi, C., Kevei, F., 2003. Isolation and characterization of a new keratinolytic *Bacillus licheniformis* strain. *World J. Microbiol. Biotechnol.* 19, 35–39.
125. Marchler-Bauer, A., Bo, Y., Han, L., He, J., Lanczycki, C.J., Lu, S., Chitsaz, F., Derbyshire, M.K., Geer, R.C., Gonzales, N.R., Gwadz, M., Hurwitz, D.I., Lu, F., Marchler, G.H., Song, J.S., Thanki, N., Wang, Z., Yamashita, R.A., Zhang, D., Zheng, C., Geer, L.Y., Bryant, S.H., 2017. CDD/SPARCLE: Functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res.* 45, D200–D203.
126. Marshall, R.C., Orwin, D.F.G., Gillespie, J.M., 1991. Structure and biochemistry of mammalian hard keratin. *Electron Microsc. Rev.* 4, 47–83.
127. Martínez-Alvarez, O., Chamorro, S., Brenes, A., 2015. Protein hydrolysates from animal processing by-products as a source of bioactive molecules with interest in animal feeding: A review. *Food Res. Int.* 73, 204–212.

128. Mazotto, A.M., Ramírez Ascheri, J.L., de Oliveira Godoy, R.L., Triches Damaso, M.C., Couri, S., Vermelho, A.B., 2017. Production of feather protein hydrolyzed by *B. subtilis* AMR and its application in a blend with cornmeal by extrusion. *LWT - Food Sci. Technol.* 84, 701–709.
129. McCasland, W.E., Richardson, L.R., 1966. Methods for determining the nutritive value of feather meals. *Poult. Sci.* 45, 1231–1236.
130. McKittrick, J., Chen, P.-Y., Bodde, S.G., Yang, W., Novitskaya, E.E., Meyers, M.A., 2012. The structure, functions, and mechanical properties of keratin. *JOM.* 64, 449–468.
131. Meng, K., Li, J., Cao, Y., Shi, P., Wu, B., Han, X., Bai, Y., Wu, N., Yao, B., 2007. Gene cloning and heterologous expression of a serine protease from *Streptomyces fradiae* var.k11. *Can. J. Microbiol.* 53, 186–195.
132. Mitsuiki, S., Ichikawa, M., Oka, T., Sakai, M., Moriyama, Y., Sameshima, Y., Goto, M., Furukawa, K., 2004. Molecular characterization of a keratinolytic enzyme from an alkaliphilic *Nocardiopsis* sp. TOA-1. *Enzyme Microb. Technol.* 34, 482–489.
133. Mitsuiki, S., Sakai, M., Moriyama, Y., Goto, M., Furukawa, K., 2002. Purification and Some Properties of a Keratinolytic Enzyme from an *Alkaliphilic Nocardiopsis* sp. TOA-1. *Biosci. Biotechnol. Biochem.* 66, 164–167.
134. Modenbach, A.A., Nokes, S.E., 2013. Enzymatic hydrolysis of biomass at high-solids loadings-a review. *Biomass Bioenergy* 56, 526–544.
135. Morán, E.T., Bayley, H.S., Summers, J.D., 1967. Keratins as source of protein for the growing chick. 3. The metabolizable energy and amino acid composition of raw and processed hog hair meal with emphasis on cystine destruction with autoclaving. *Poult. Sci.* 46, 548–553.
136. Moreira-Gasparin, F.G., de Souza, C.G.M., Costa, A.M., Alexandrino, A.M., Bracht, C.K., Boer, C.G., Peralta, R.M., 2009. Purification and characterization of an efficient poultry feather degrading-protease from *Myrothecium verrucaria*. *Biodegradation* 20, 727–736.
137. Morup, I.L.K., Olesen, E.S., 1976. New method for prediction of protein value from essential amino acid pattern. *Nutr. Rep. Int.* 13, 355–365.
138. Nam, G.W., Lee, D.W., Lee, H.S., Lee, N.J., Kim, B.C., Choe, E.A., Hwang, J.K., Suhartono, M.T., Pyun, Y.R., 2002. Native-feather degradation by *Fervidobacterium islandicum* AW-1, a newly isolated keratinase-producing thermophilic anaerobe. *Arch. Microbiol.* 178, 538–547.

139. Ni, H., Chen, Q., Chen, F., Fu, M., Dong, Y., Cai, H., 2011. Improved keratinase production for feather degradation by *Bacillus licheniformis* ZJUEL31410 in submerged cultivation. *African J. Biotechnol.* 10, 7236–7244.
140. Ningthoujam, D., Devi, L., Devi, P., Kshetri, P., Tamreihao, K., Mukherjee, S., Devi, S., Betterson, N., 2016. Optimization of Keratinase Production by *Amycolatopsis* sp . Strain MBRL 40 from a Limestone Habitat. *J. Bioprocess Biotech.* 6, 282.
141. Palmqvist, B., Lidén, G., 2012. Torque measurements reveal large process differences between materials during high solid enzymatic hydrolysis of pretreated lignocellulose. *Biotechnol Biofuels.* 5, 57–65.
142. Papadopoulos, M.C., 1985. Processed chicken feathers as feedstuff for poultry and swine. A review. *Agric. Wastes* 14, 275–290.
143. Papadopoulos, M.C., El-Boushy, A.R., Roodbeen, A.E., 1985. The effect of varying autoclaving conditions and added sodium hydroxide on amino acid content and nitrogen characteristics of feather meal. *J. Sci. Food Agric.* 36, 1219–1226.
144. Pastor-Cavada, E., Juan, R., Pastor, J.E., Alaiz, M., Vioque, J., 2011. Nutritional characteristics of seed proteins in 15 Lathyrus species (fabaceae) from Southern Spain. *LWT - Food Sci. Technol.* 44, 1059–1064.
145. Pasupuleti, V.K., Holmes, C., Demain, A.L., 2010. Applications of protein hydrolysates in biotechnology, in: *Protein Hydrolysates in Biotechnology*. pp. 1–9.
146. Paul, T., Das, A., Mandal, A., Halder, S.K., DasMohapatra, P.K., Pati, B.R., Mondal, K.C., 2014b. Valorization of Chicken Feather Waste for Concomitant Production of Keratinase, Oligopeptides and Essential Amino Acids Under Submerged Fermentation by *Paenibacillus woosongensis* TKB2. *Waste and Biomass Valorization* 5, 575–584.
147. Paul, T., Das, A., Mandal, A., Jana, A., Maity, C., Adak, A., Halder, S.K., DasMohapatra, P.K., Pati, B.R., Mondal, K.C., 2014a. Effective dehairing properties of keratinase from *Paenibacillus woosongensis* TKB2 obtained under solid state fermentation. *Waste and Biomass Valorization* 5, 97–107.
148. Paul, T., Jana, A., Mandal, A.K., Mandal, A., Das Mohapatra, P.K., Mondal, K.C., 2016. Bacterial keratinolytic protease, imminent starter for NextGen leather and detergent industries. *Sustain. Chem. Pharm.* 3, 8–22.

149. Pawar, V.A., Prajapati, A.S., Akhiani, R.C., Patel, D.H., Subramanian, R.B., 2018. Molecular and biochemical characterization of a thermostable keratinase from *Bacillus altitudinis* RBDV1. 3 Biotech 8.
150. Pearce, K.E.N., Karahalios, D.A., Friedman, M.E., 1988. Ninhydrin Assay For Proteolysis in Ripening Cheese. J. Food Sci. 53, 432–435.
151. Perona, J.J., Craik, C.S., 1997. Evolutionary divergence of substrate specificity within the chymotrypsin-like serine protease fold. J. Biol. Chem. 272, 29987–29990.
152. Petersen, T.N., Brunak, S., Von Heijne, G., Nielsen, H., 2011. SignalP 4.0: Discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786.
153. Pleissner, D., Venus, J., 2016. Utilization of protein-rich residues in biotechnological processes. Appl. Microbiol. Biotechnol. 100, 2133–2140.
154. Prakash, P., Jayalakshmi, S.K., Sreeramulu, K., 2010a. Production of keratinase by free and immobilized cells of *Bacillus halodurans* Strain PPKS-2: Partial characterization and its application in feather degradation and dehairing of the goat skin. Appl. Biochem. Biotechnol. 160, 1909–1920.
155. Prakash, P., Jayalakshmi, S.K., Sreeramulu, K., 2010b. Purification and characterization of extreme alkaline, thermostable keratinase, and keratin disulfide reductase produced by *Bacillus halodurans* PPKS-2. Appl. Microbiol. Biotechnol. 87, 625–633.
156. Rahayu, S., Syah, D., Suhartono, M.T., 2012. Degradation of keratin by keratinase and disulfide reductase from *Bacillus* sp. MTS of Indonesian origin. Biocatal Agric Biotechnol. 1, 152–158.
157. Rai, S.K., Konwarh, R., Mukherjee, A.K., 2009. Purification, characterization and biotechnological application of an alkaline β -keratinase produced by *Bacillus subtilis* RM-01 in solid-state fermentation using chicken-feather as substrate. Biochem. Eng. J. 45, 218–225.
158. Rai, S.K., Mukherjee, A.K., 2011. Optimization of production of an oxidant and detergent-stable alkaline β -keratinase from *Brevibacillus* sp. strain AS-S10-II: Application of enzyme in laundry detergent formulations and in leather industry. Biochem. Eng. J. 54, 47–56.
159. Ramakrishna Reddy, M., Sathi Reddy, K., Ranjita Chouhan, Y., Bee, H., Reddy, G., 2017. Effective feather degradation and keratinase production by *Bacillus pumilus* GRK for its application as bio-detergent additive. Bioresour. Technol. 243, 254–263.

160. Ramnani, P., Gupta, R., 2004. Optimization of medium composition for keratinase production on feather by *Bacillus licheniformis* RG1 using statistical methods involving response surface methodology. *Biotechnol. Appl. Biochem.* 40, 191.
161. Ramnani, P., Gupta, R., 2007. Keratinases vis-à-vis conventional proteases and feather degradation. *World J. Microbiol. Biotechnol.* 23, 1537–1540.
162. Ramnani, P., Singh, R., Gupta, R., 2005. Keratinolytic potential of *Bacillus licheniformis* RG1: structural and biochemical mechanism of feather degradation. *Can. J. Microbiol.* 51, 191–196.
163. Reddy, M.R., Reddy, K.S., Chouhan, Y.R., Bee, H., Reddy, G., 2017. Effective feather degradation and keratinase production by *Bacillus pumilus* GRK for its application as bio-detergent additive. *Bioresour. Technol.* 243, 254–263.
164. Ren, M., Habte-Tsion, H.M., Liu, B., Miao, L., Ge, X., Xie, J., Liang, H., Zhou, Q., Pan, L., 2015. Dietary leucine level affects growth performance, whole body composition, plasma parameters and relative expression of TOR and TNF- α in juvenile blunt snout bream, *Megalobrama amblycephala*. *Aquaculture* 448, 162–168.
165. Riessen, S., Antranikian, G., 2001. Isolation of *Thermoanaerobacter keratinophilus* sp. nov., a novel thermophilic, anaerobic bacterium with keratinolytic activity. *Extremophiles* 5, 399–408.
166. Riffel, A., Brandelli, A., Bellato, C. de M., Souza, G.H.M.F., Eberlin, M.N., Tavares, F.C.A., 2007. Purification and characterization of a keratinolytic metalloprotease from *Chryseobacterium* sp. kr6. *J. Biotechnol.* 128, 693–703.
167. Riffel, A., Daroit, D.J., Brandelli, A., 2011. Nutritional regulation of protease production by the feather-degrading bacterium *Chryseobacterium* sp. kr6. *N. Biotechnol.* 28, 153–157.
168. Robbins, C.R., 2012. Chemical and physical behavior of human hair. fifth ed. Springer-Verlag, Berlin Heidelberg.
169. Rozs, M., Manczinger, L., Vágvölgyi, C., Kevei, F., 2001. Secretion of a trypsin-like thiol protease by a new keratinolytic strain of *Bacillus licheniformis*. *FEMS Microbiol. Lett.* 205, 221–224.
170. Ruffin, P., Andrieu, S., Biserte, G., Biguet, J., 1976. Sulphitolysis in keratinolysis. Biochemical proof. *Sabouraudia*. 14, 181–184.

171. Sahoo, D.K., Das, A., Thatoi, H., Mondal, K.C., Mohapatra, P.K.D., 2012. Keratinase production and biodegradation of whole chicken feather keratin by a newly isolated bacterium under submerged fermentation. *Appl. Biochem. Biotechnol.* 167, 1040–1051.
172. Sahoo, D.K., Thatoi, H.N., Mitra, B., Mondal, K.C., Das Mohapatra, P.K., 2017. Advances in Microbial Keratinase and Its Potential Applications, in: Patra, J., Vishnuprasad, C., Das, G. (Eds.), *Microbial Biotechnology*. Springer, Singapore, pp. 105–133.
173. Sanyal, A.K., Das, S.K., Banerjee, A.B., 1985. Purification and partial characterization of an exocellular proteinase from *Trichophyton rubrum*. *Sabouraudia* 23, 165–78.
174. Sattler, W., Esterbauer, H., Glatter, O., Steiner, W., 1989. The effect of enzyme concentration on the rate of the hydrolysis of cellulose. *Biotechnol. Bioeng.* 33, 1221–1234.
175. Schonbaum, G., Zerner, B., Bender, M., 1961. The Spectrophotometric Determination of the Operational Normality of an α -Chymotrypsin Solution. *J. Biol. Chem.* 236, 2930–2935.
176. Shamushaki, V.A.J., Kasumyan, A.O., Abedian, A., Abtahi, B., 2007. Behavioural responses of the Persian sturgeon (*Acipenser persicus*) juveniles to free amino acid solutions. *Mar. Freshw. Behav. Physiol.* 40, 219–224.
177. Sharma, R., Devi, S., 2018. Versatility and commercial status of microbial keratinases: a review. *Rev. Environ. Sci. Biotechnol.* 17, 19–45.
178. Sharma, R., Gupta, R., 2012. Coupled action of γ -glutamyl transpeptidase-glutathione and keratinase effectively degrades feather keratin and surrogate prion protein, Sup 35NM. *Biore-sour. Technol.* 120, 314–317.
179. Sindayikengera, S., Xia, W., 2006. Nutritional evaluation of caseins and whey proteins and their hydrolysates from Protamex. *J. Zhejiang Univ. Sci. B* 7, 90–98.
180. Sinkiewicz, I., Staroszczyk, H., Śliwińska, A., 2018. Solubilization of keratins and functional properties of their isolates and hydrolysates. *J. Food Biochem.* e12494.
181. Sipos, T., Merkel, J.R., 1970. An Effect of Calcium Ions on the Activity, Heat Stability, and Structure. *Biochemistry* 9, 2766–2775.
182. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of Protein Using Bicinchoninic Acid. *Anal. Biochem.* 19, 76–85.
183. Sonenshein, A.L., 2005. CodY, a global regulator of stationary phase and virulence in Gram-positive bacteria. *Curr. Opin. Microbiol.* 8, 203–207.

184. Stiborova, H., Branska, B., Vesela, T., Lovecka, P., Stranska, M., Hajslova, J., Jiru, M., Patakova, P., Demnerova, K., 2016. Transformation of raw feather waste into digestible peptides and amino acids. *J. Chem. Technol. Biotechnol.* 91, 1629–1637.
185. Suntornsuk, W., Suntornsuk, L., 2003. Feather degradation by *Bacillus* sp. FK 46 in submerged cultivation. *Bioresour. Technol.* 86, 239–43.
186. Takeuchi, T., (2007). Amino acids, Peptides, in: Nakagawa, H., Sato, M., Gatlin, D.M., (Eds.), *Dietary supplements for the health and quality of cultured fish*. CAB International, Oxon, UK, pp. 47–63.
187. Taskin, M., Kurbanoglu, E.B., 2011. Evaluation of waste chicken feathers as peptone source for bacterial growth. *J. Appl. Microbiol.* 111, 826–834.
188. Tatineni, R., Doddapaneni, K.K., Potumarthi, R.C., Vellanki, R.N., Kandathil, M.T., Kolli, N., Mangamoori, L.N., 2008. Purification and characterization of an alkaline keratinase from *Streptomyces* sp. *Bioresour. Technol.* 99, 1596–1602.
189. Tervasmäki, P., Sotaniemi, V., Kangas, J., Taskila, S., Ojamo, H., Tanskanen, J., 2017. A discretized model for enzymatic hydrolysis of cellulose in a fed-batch process. *Bioresour. Technol.* 227, 112–124.
190. Thys, R.C.S., Brandelli, A., 2006. Purification and properties of a keratinolytic metalloprotease from *Microbacterium* sp. *J. Appl. Microbiol.* 101, 1259–1268.
191. Tiwary, E., Gupta, R., 2010. Medium optimization for a novel 58kDa dimeric keratinase from *Bacillus licheniformis* ER-15: Biochemical characterization and application in feather degradation and dehairing of hides. *Bioresour. Technol.* 101, 6103–6110.
192. Tiwary, E., Gupta, R., 2012. Rapid Conversion of Chicken Feather to Feather Meal Using Dimeric Keratinase from *Bacillus licheniformis* ER-15. *J. Bioprocess. Biotech.* 2, 1–5.
193. Tork, S.E., Shahein, Y.E., El-Hakim, A.E., Abdel-Aty, A.M., Aly, M.M., 2016. Purification and partial characterization of serine-metallokeratinase from a newly isolated *Bacillus pumilus* NRC21. *Int. J. Biol. Macromol.* 86, 189–196.
194. Verma, A., Singh, H., Anwar, S., Chattopadhyay, A., Tiwari, K.K., Kaur, S., Dhilon, G.S., 2017. Microbial keratinases: industrial enzymes with waste management potential. *Crit. Rev. Biotechnol.* 37, 476–491.
195. Vignardet, C., Guillaume, Y.C., Michel, L., Friedrich, J., Millet, J., 2001. Comparison of two hard keratinous substrates submitted to the action of a keratinase using an experimental design. *Int J Pharm.* 224, 115–122.

196. Wan, M.-Y., Dong, G., Yang, B.-Q., Feng, H., 2016. Identification and characterization of a novel antioxidant peptide from feather keratin hydrolysate. *Biotechnol. Lett.* 38, 643–649.
197. Wang, B., Yang, W., Mckittrick, J., Meyers, M.A., 2016. Keratin: Structure, mechanical properties, occurrence in biological organisms, and efforts at bioinspiration. *Prog Mater Sci.* 76, 229–318.
198. Wang, J.J., Shih, J.C.H., 1999. Fermentation production of keratinase from *Bacillus licheniformis* PWD-1 and a recombinant *B. subtilis* FDB-29. *J. Ind. Microbiol. Biotechnol.* 22, 608–616.
199. Wang, J.J., Swaisgood, H.E., Shih, J.C.H., 2003. Production and characterization of bio-immobilized keratinase in proteolysis and keratinolysis. *Enzyme Microb. Technol.* 32, 812–819.
200. Wang, S.-L., Hsu, W.-T., Liang, T.-W., Yen, Y.-H., Wang, C.-L., 2008. Purification and characterization of three novel keratinolytic metalloproteases produced by *Chryseobacterium indologenes* TKU014 in a shrimp shell powder medium. *Bioresour. Technol.* 99, 5679–5686.
201. Wang, X., Parsons, C.M., 1997. Effect of processing systems on protein quality of feather meals and hog hair meals. *Poult. Sci.* 76, 491–496.
202. Williams, C.M., Lee, C.G., Garlich, J.D., Shih, J.C.H., 1991. Evaluation of a Bacterial Feather Fermentation Product, Feather-Lysate, as a Feed Protein. *Poult. Sci.* 70, 85–94.
203. Wilson, R., 2002. Amino Acids and Proteins, in: *Fish Nutrition*. Elsevier, pp. 144–175.
204. Wu, W.L., Chen, M.Y., Tu, I.F., Lin, Y.C., Eswarkumar, N., Chen, M.Y., Ho, M.C., Wu, S.H., 2017. The discovery of novel heat-stable keratinases from *Meiothermus taiwanensis* WR-220 and other extremophiles. *Sci. Rep.* 7, 4658.
205. Xu, H., Yang, Y., 2014. Controlled de-cross-linking and disentanglement of feather keratin for fiber preparation via a novel process. *ACS Sustain. Chem. Eng.* 2, 1404–1410.
206. Yamamura, S., Morita, Y., Hasan, Q., Yokoyama, K., Tamiya, E., 2002b. Keratin degradation: a cooperative action of two enzymes from *Stenotrophomonas sp.* *Biochem. Biophys. Res. Commun.* 294, 1138–1143.
207. Yamamura, S., Ramachandra, S., Yokoyama, K., 2002a. Characterization of a New Keratin-Degrading Isolated from Deer Fur Bacterium. *J. Biosci. Bioeng.* 93, 595–600.
208. Yang, L., Wang, H., Lv, Y., Bai, Y., Luo, H., Shi, P., Huang, H., Yao, B., 2016. Construction of a rapid feather-degrading bacterium by overexpression of a highly efficient alkaline keratinase in its parent strain *Bacillus amyloliquefaciens* K11. *J. Agric. Food Chem.* 13, 78–84.

209. Yusuf, I., Ahmad, S.A., Phang, L.Y., Syed, M.A., Shamaan, N.A., Abdul Khalil, K., Dahalan, F.A., Shukor, M.Y., 2016. Keratinase production and biodegradation of polluted secondary chicken feather wastes by a newly isolated multi heavy metal tolerant bacterium-*Alcaligenes* sp. AQ05-001. *J. Environ. Manage.* 183, 182–195.
210. Zaghloul, T.I., Embaby, A.M., Elmahdy, A.R., 2011a. Key determinants affecting sheep wool biodegradation directed by a keratinase-producing *Bacillus subtilis* recombinant strain. *Biodegradation* 22, 111–128.
211. Zaghloul, T.I., Embaby, A.M., Elmahdy, A.R., 2011b. Biodegradation of chicken feathers waste directed by *Bacillus subtilis* recombinant cells: Scaling up in a laboratory scale fermentor. *Bioresour. Technol.* 102, 2387–2393.
212. Zhang, Y., Dabrowski, K., Hliwa, P., Gomulka, P., 2006. Indispensable amino acid concentrations decrease in tissues of stomachless fish, common carp in response to free amino acid- or peptide-based diets. *Amino Acids* 31, 165–172.
213. Zhao, Y., Hu, Y., Zhou, X.-Q., Zeng, X.-Y., Feng, L., Liu, Y., Jiang, W.-D., Li, S.-H., Li, D.-B., Wu, X.-Q., Wu, C.-M., Jiang, J., 2015. Effects of dietary glutamate supplementation on growth performance, digestive enzyme activities and antioxidant capacity in intestine of grass carp (*Ctenopharyngodon idella*). *Aquac. Nutr.* 21, 935–941.
214. Zhao, Y., Xiang, S., Dai, X., Yang, K., 2013. A simplified diphenylamine colorimetric method for growth quantification. *Appl Microbiol Biotechnol.* 97, 5069–5077.

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